TABLE 1 Effects of LPS and TNF-α on TNF-R2$^{-/-}$ mice

Dose			Lethality	
LPS (μg)	D-galactosamine (mg)*	TNF-α† (μg)	TNF-R2$^{+/+}$	TNF-R2$^{-/-}$
800	—	—	5/5	5/5
600	—	—	9/10	4/10
500	—	—	5/5	2/5
300	—	—	3/4	1/4
100	—	—	0/4	0/4
100	20	—	5/5	5/5
10	20	—	5/5	5/5
1.0	20	—	5/5	5/5
0.1	20	—	9/10	9/10
0.01	20	—	6/7	6/7
0.001	20	—	1/5	2/5
		10	10/10	1/11
		15	4/4	4/4

* Mice were injected intraperitoneally with the indicated amount of LPS (*Salmonella* abortus equi; Sigma) with or without D-Galactosamine (20 mg per mouse) in 0.2 ml Hank's balanced salt solution. Lethality was monitored over 3 days and indicated as lethality/total injected. *P* values were <0.006 for LPS toxicity and <0.01 for the TNF study.

† Recombinant murine TNF-α (10 μg; Genentech) was injected intravenously.

following TNF injection also demonstrates the decreased sensitivity to TNF in TNF-R2$^{-/-}$ mice. This decreased sensitivity is consistent with the 'ligand passing' model in which TNF-R2 helps recruit TNF for interaction with TNF-R1 and lowers the concentration of TNF needed for TNF-R1 signal transduction[19]. Alternatively, TNF-R2 could synergize with TNF-R1 in transducing the TNF signal, or TNF-R2 could perform other functions in this system that cause additional damage. Neither TNF-R1- nor TNF-R2-deficient mice are completely insensitive to high doses of LPS alone, but both show decreased sensitivity to TNF. Thus, either both receptors contribute to LPS-induced septic shock or other cytokines produced during sepsis act independently of TNF and contribute greatly to toxicity. Interbreeding of TNF-R1$^{-/-}$ and TNF-R2$^{-/-}$ mice to generate double-receptor-ablated mice should help clarify this issue. Strategies for therapeutic intervention of sepsis may be influenced by these results. □

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Activation of the kinase Pelle by Tube in the dorsoventral signal transduction pathway of *Drosophila* embryo

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THE concentration of Dorsal protein in the nucleus determines cell fate along the dorsoventral axis of the *Drosophila* embryo[1–13]. The *dorsal*-group genes and the *cactus* gene are required for production and transmission of a localized signal on the ventral side of the embryo[4,5] which determines the position of the highest nuclear concentration of Dorsal protein[1–3]. The ventralizing signal produced in somatic cells[6] is transmitted through the perivitelline space[7] to the integral membrane protein Toll[8]. Inside the embryo it leads to dissociation of the cytoplasmic Dorsal–Cactus complex and subsequent nuclear localization of Dorsal protein[9,10]. Two components are known to mediate the signal transduction between Toll and Dorsal–Cactus[11,12]: Pelle, a serine/threonine protein kinase[13], and Tube, a protein with an unknown biochemical activity[14]. Here we construct gain-of-function alleles of *pelle* and *tube* and show that *pelle* functions downstream of *tube*. In addition, Pelle and Tube interact directly with one another. We propose that Tube is a direct activator of the protein kinase Pelle.

In dorsalized embryos, Dorsal protein is restricted to the cytoplasm, whereas in ventralized embryos all nuclei around the egg circumference contain Dorsal protein[1–3]. Analysis of double mutants combining a ventralizing allele of *Toll* and a dorsalizing allele of the *dorsal*-group genes established their functional order. It has been shown that only *pelle*, *tube* and *dorsal* function downstream of *Toll*, and the remaining *dorsal*-group genes (such as *snake* and *nudel*) function upstream[4,12]. But the functional order of *pelle* and *tube* was unclear as no ventralizing alleles for these genes were available. We constructed ventralizing gain-of-function alleles of *pelle* and *tube* by replacing the intracellular kinase domain of a gain-of-function allele of the receptor tyrosine kinase Torso, *torso*^{4021} (refs 15, 16), by the *pelle* or *tube* coding sequences. We refer to these constructs as *tor*^{4021}*pelle* and *tor*^{4021}*tube*. A similar strategy has been used to obtain an activated form of *D-raf* in the investigation of the *sevenless* signal transduction pathway[17]. RNA encoding the fusion proteins was synthesized *in vitro* and microinjected into embryos from females of various genotypes.

Injection of *tor*^{4021}*pelle* RNA into embryos from *pelle* females, and *tor*^{4021}*tube* RNA into embryos from *tube* females induced dorsolateral and ventrolateral pattern elements. A similar effect was observed when *tor*^{4021}*pelle* or *tor*^{4021}*tube* RNA was injected into embryos from females mutant for the upstream *dorsal*-group gene *snake*. Near the injection site, embryos developed ventral denticle belts and filzkörper (Table 1 and Fig. 1*a*, *c*, *d*) which are absent in mutant embryos (Fig. 1*b*). In addition, expression of *twist* (Fig. 1*e*) was induced at the injection site, indicating the highest levels of nuclear Dorsal protein[1]. To confirm the gain-of-function effect, we analysed the position of the posterior midgut invagination, which normally occurs on the dorsal side. When RNA was deposited on the dorsal side of the egg, we observed a posterior midgut invagination at a ventral position, opposite the site of RNA deposition (Fig. 1*f–h*), indicating a clear reversal of dorsoventral polarity. There is no strict requirement for the *torso*^{4021} allele, as fusion constructs with

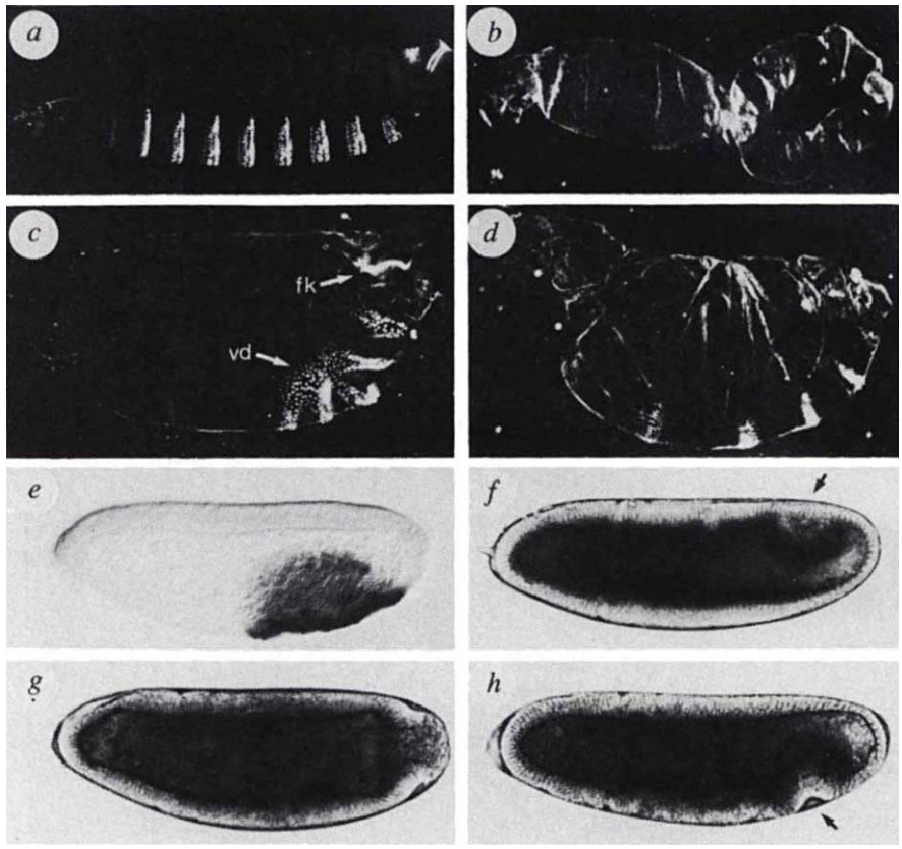

FIG. 1 Induction of dorsoventral pattern elements by *tor*^{4021}*pelle* RNA. *a–d*, Cuticle preparations of embryos from wild-type (*a*), *tube* (*b*, *d*) and *snake* (*c*) females. *a* and *b*, Not injected; *c* and *d*, injected with *tor*^{4021}*pelle* RNA. Filzkörper (fk) and ventral denticle belts (vd) are indicated. These structures are absent in completely dorsalized embryos (*b*). Fusion to *torso* is necessary for activation, as unmodified *pelle* and *tube* RNA had no effect when injected into embryos from *snake* females (data not shown). Similarly, *torso*^{4021} RNA injected into embryos from *snake* females did not induce filzkörper (data not shown). *e*, An embryo from a *tube* female injected with *tor*^{4021}*pelle3′* RNA (see below) and stained for Twist (antibody provided by R. Reuter). *f–h*, Living embryos from *snake* females, uninjected (*g*) or injected with *tor*^{4021}*pelle* RNA either dorsally (*h*) or ventrally (*f*). The position of posterior midgut invagination is indicated by arrows.

METHODS. The *tor*^{4021}*pelle* fusion construct with amino-acid residues 1–455 of Torso^{4021} (refs 16, 26) and 2–501 of Pelle[13] contains the signal peptide, the extracellular domain, and the transmembrane peptide of Torso, with Pelle as its intracellular domain. In the *torso*^{4021} allele, a single amino-acid residue is changed in the extracellular domain[26]. DNA encoding Pelle, with *Bst*EII and *Xho*I sites, was prepared by polymerase chain reaction (PCR) and cloned into the vector pB4021 (ref. 26) missing a *Bst*EII–*Sal*I fragment. The related construct *tor*^{4021}*pelle3′*, which contains the 3′ untranslated region of the *pelle* cDNA in addition to the coding sequence, was constructed similarly. RNA was synthesized *in vitro* and injected as described[26] at about 1 mg ml^{-1} into the dorsal or ventral cortical zone in the posterior half of embryos, at about 30% egg length, before pole cell formation. The allelic combinations *tub*$^{R5.6}$/*Df(3R)hkb*^{XM3} and *snk*^{073}/*snk*^{229} were used.

FIG. 2 Pelle and Tube interact physically. *a*, Interaction in the two-hybrid system. Yeast strains containing a *LEU2* and a *lacZ* reporter gene, and plasmids encoding the indicated combination of lexA and activation-tagged fusion proteins, were streaked out on synthetic medium without leucine and incubated for four days. The assay for *lacZ* reporter gene activation gave a similar result (data not shown). The slow growth of some yeast strains containing the lexA–pelle fusion protein is due to the property of LexA–pelle to activate the reporter gene weakly by itself, as seen in the combination lexA–pelle/JG4-5. *b*, Association *in vitro*. Indicated combinations of radiolabelled and unlabelled protein were subjected to immunoprecipitation with an antibody specific for the unlabelled protein, and analysed by SDS–PAGE (upper panel, immunoprecipitates (IP), lower panel, supernatants (SN)). *c*, Pelle phosphorylates Tube *in vitro*. Pelle immunoprecipitates prepared from wild-type (OR) and *pelle* embryonic extracts (rm8, 019) were assayed for their ability to phosphorylate Tube *in vitro* (upper panel). Presence of Pelle was assayed by western blotting of total embryonic extracts (lower panel). *d*, Phosphorylation of bacterially expressed Tube, Cactus and Dorsal protein by Pelle immunoprecipitates prepared from *nudel* (ndl) and *Toll* (Tl^{10b}) embryonic extracts, followed by SDS–PAGE (upper panel, short exposure; middle panel, long exposure of the same gel). Pelle immunoprecipitates were omitted in the buffer controls. Lower panel, autophosphorylation of Pelle immunoprecipitates.

METHODS. DNA containing the coding sequences of the indicated genes[8,13,14,19,23,27,28] (except toll^{in} with codons 830–1097) and appropriate ends for cloning was prepared by PCR and cloned into the vectors pEG202 and pJG4-5 in the reading frames of lexA and of the activation tag, respectively[29]. Yeast strains contained a chromosomal *LEU2* reporter gene (EGY48) (ref. 29) and the *lacZ* reporter gene pSH18–34 (constructed by S. Hanes). Assays for reporter gene activation were done on synthetic galactose/raffinose medium without leucin. Cloning, expression in *Escherichia coli*, and purification of 6 × His-tagged Tube[14], Pelle[13], Cactus and Dorsal[23] proteins were done with the vectors pQE12 and pQE30 (Qiagen). Production and affinity purification of Tube-, Pelle-, Cactus- and Dorsal-specific antibodies and western blot analysis were modified from refs 1 and 10. Reticulocyte lysate (5 µl) (Amersham) containing ^{35}S-labelled protein was incubated with 100 ng bacterially expressed, purified protein (6 × His-tagged Tube, Cactus, Dorsal proteins; bacterial control extract pQE12) for 30 min at 25 °C. Immunoprecipitation was done with 2 µg of an antibody specific for the unlabelled protein, pre-bound to protein A–Sepharose beads (Pharmacia) in immunoprecipitation (IP) buffer (50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, with protease and phosphatase inhibitors) for 2 h at 4 °C followed by five washing steps with buffer, and SDS–PAGE. Protein in the supernatants was precipitated with trichloroacetic acid and subjected to SDS–PAGE. Embryonic extracts were obtained by lysing 0–4-h embryos of the indicated maternal genotypes (*pll*^{rm8}/*Df(3R)D605*, *pll*^{019}/*Df(3R)D605*, *ndl*^{046}/*ndl*^{093}, *Tl*^{10b}/+) in IP buffer. In protein kinase assays, Pelle immunoprecipitates were incubated with 100 ng bacterially expressed, purified 6 × His-tagged Tube, Cactus or Dorsal protein in 30 µl kinase buffer[30] for 30 min at 25 °C. After centrifugation, Pelle immunocomplexes were washed four times with IP buffer and used for autophosphorylation analysis. Supernatants were immunoprecipitated with either Tube-, Cactus- or Dorsal-specific antibodies. Analysis was by SDS–PAGE and fluorography. The gel in *d* was exposed overnight without an intensifying screen (upper and lower panels) or for three days with an intensifying screen (middle).

the corresponding part of wild-type *torso* show similar effects, although their activity is weaker (data not shown). Thus, fusions of *pelle* and *tube* with *torso* bypass the normal requirement for upstream *dorsal*-group genes for the induction of dorsoventral pattern elements and are able to induce polarity independently of the prelocalized signal.

The ventralizing constructs, tor^{4021}*pelle* and tor^{4021}*tube*, were used to determine the relative position of the two gene functions in the signalling pathway. Injection of tor^{4021}*pelle* RNA into embryos from *tube* females resulted in the same rescue response as injection into embryos from *snake* or *pelle* females (Fig. 1*c*, *d* and Table 1). In contrast, we observed no effect of injection of tor^{4021}*tube* RNA into embryos from *pelle* females (Table 1). Neither of the fusion constructs was able to induce dorsoventral pattern elements in embryos from *dorsal* females (Table 1), consistent with previous findings that *dorsal* is the final target of the dorsoventral signal transduction pathway[4,11]. As disruption of *pelle* activity blocks signalling by tor^{4021}*tube*, whereas on the

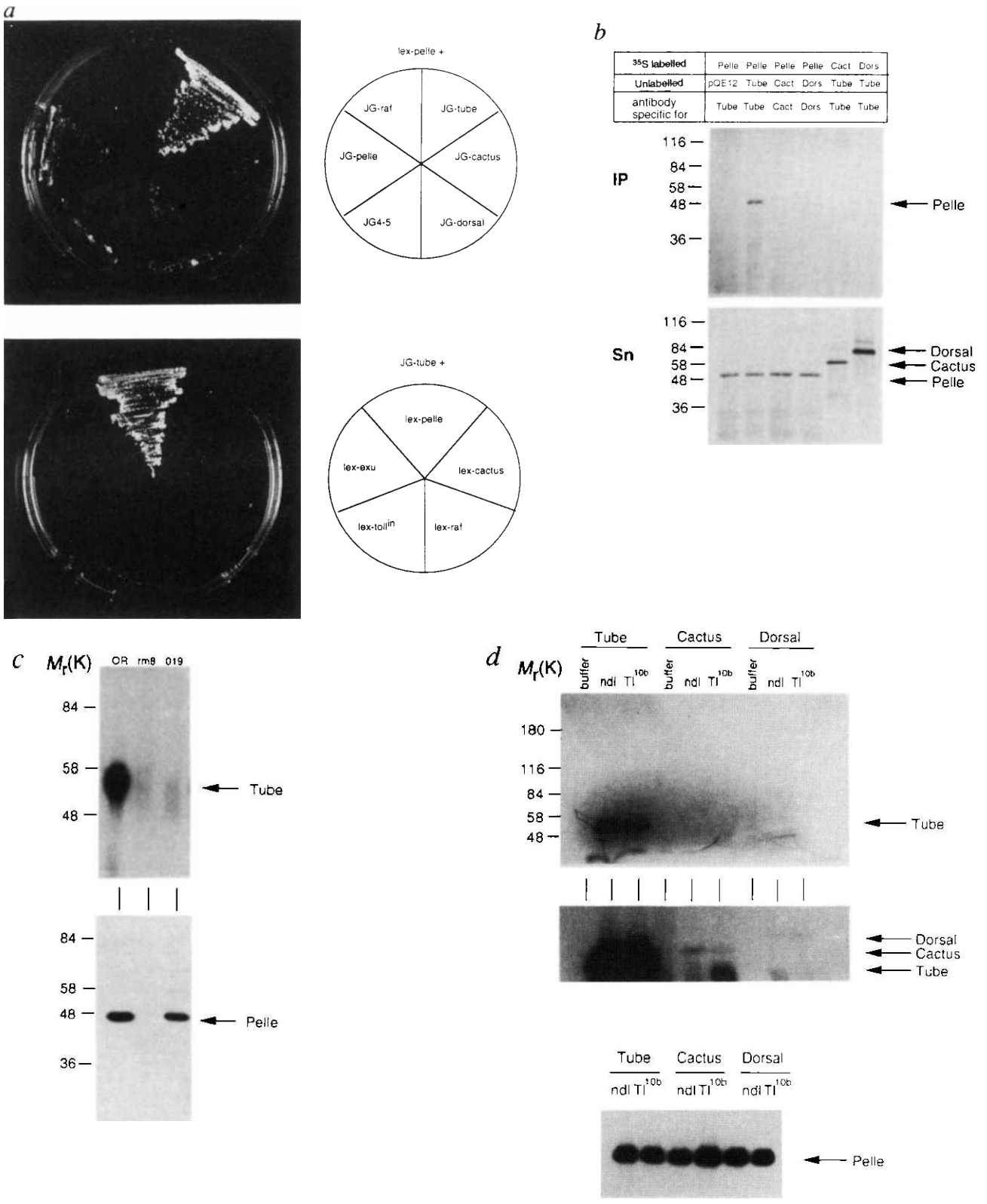

TABLE 1 *tor*^{4021}*pelle* RNA and *tor*^{4021}*tube* RNA injections

RNA injected (1 mg ml^{-1})	Maternal genotype	Number of differentiated embryos	Cuticle phenotype				Partial rescue (FK or VD)
			DO	FK	FK + VD	VD	
tor^{4021}*pelle*	*snake*	114	41	46	22	5	64%
	tube	77	31	32	14	0	62%
	pelle	42	14	6	15	7	66%
	dorsal	108	105	(3)	0	0	0%
tor^{4021}*tube*	*snake*	111	18	15	29	49	84%
	tube	48	5	3	11	29	90%
	pelle	36	36	0	0	0	0%
	dorsal	29	29	0	0	0	0%

tor^{4021}*pelle* and *tor*^{4021}*tube* RNA (about 1 mg ml^{-1}) were injected into embryos from females of the indicated genotypes before pole-cell formation. RNA was deposited either in the dorsal or ventral cortical zone at about 30% egg length. There was no difference in frequency of rescue between embryos injected dorsally and ventrally. Cuticle preparations of differentiated embryos were scored according to the following categories: completely dorsalized (DO), complete filzkörper or filzkörper material present (FK), complete or partial ventral denticle belts present (VD), and filzkörper or filzkörper material and ventral denticle belts present (FK + VD). Allelic combinations used: *snk*^{073}/*snk*^{229}, *tub*$^{R5.6}$/*Df(3R)hkb*^{XM3}, *pll*^{rm8}/*pll*^{078}, *pll*^{rm8}/*Df(3R)D605*, *dl*^{1}/*Df(2L)TW119*, *dl*^{H}/*Df(2L)TW119*. Preparation and analysis of cuticles was as described[25]. Construction of *tor*^{4021}*tube*, encoding amino-acid residues 2–462 of Tube[14], was similar to *tor*^{4021}*pelle* construction (Fig. 1 legend).

other hand *tube* is not required for function of *tor*^{4021}*pelle*, we conclude that *tube* acts upstream of *pelle* in the signalling cascade.

Certain allelic combinations of *pelle* and *tube* show dominant genetic interactions that suggest a molecular interaction[12]. We used the two-hybrid system in yeast[18] to test for a direct interaction. Pelle and Tube interact specifically, as the reporter gene was activated above basal levels (40 times higher, using the lacZ reporter gene) only in yeast strains that contained Tube and Pelle fusion proteins (Fig. 2*a*). The combinations Pelle–Dorsal and Pelle–Cactus did not activate the reporter gene, although the functional integrity of Dorsal and Cactus constructs could be shown by confirming their well established interaction[9,19,21] by the two-hybrid system (data not shown). In a preliminary screen for proteins interacting with Pelle, we isolated a *tube* complementary DNA, confirming the specificity of the Tube–Pelle interaction (unpublished observations).

To demonstrate the direct interaction of Tube and Pelle by independent means, we reconstituted their association *in vitro*. Bacterially expressed unlabelled protein was added to radiolabelled protein in reticulocyte lysate and subjected to immunoprecipitation with an antibody specific for the unlabelled protein. Of all the combinations tested, coimmunoprecipitation of Pelle was only detected when Tube and Pelle were present in the mixture (Fig. 2*b*, upper panel). Analysis of the supernatants showed that only a small proportion of Pelle was coimmunoprecipitated, indicating weak binding of the two proteins (Fig. 2*b*, lower panel).

We also observed that Tube is phosphorylated by Pelle immunoprecipitates *in vitro*. Pelle was enriched from embryonic extracts by immunoprecipitation with a Pelle-specific antibody, and incubated with bacterially expressed Tube under phosphorylation conditions. Phosphorylation of Tube is due to Pelle, because it is found with immunoprecipitates from wild-type embryos, but not with immunoprecipitates from certain *pelle* alleles (Fig. 2*c*, upper panel), which either do not contain detectable amounts of Pelle (*pll*^{rm8}), or contain a mutant form of Pelle (*pll*^{019}; Fig. 2*c*, lower panel). The phosphorylation activity is specific as no significant phosphorylation of Cactus or Dorsal protein was found under similar conditions (Fig. 2*d*, upper panel). But as faint signals of Cactus and Dorsal protein phosphorylation were visible after long exposure (Fig. 2*d*, middle panel), this experiment *in vitro* does not rule out the possibility that Cactus or Dorsal protein are direct targets of activated Pelle in the embryo[13]. Because the kinase activity in our preparation does not depend on the ventralizing signal (compare *ndl* dorsalized with *Tl*^{10b} ventralized extracts; Fig. 2*d*), and Tube acts upstream of Pelle, the significance of a Pelle-mediated phosphorylation of Tube *in vivo* is not clear. A phosphorylation of Tube in the embryo could be part of a feedback mechanism regulating signal transduction between Tube and Pelle. Although it might not play a role *in vivo*, we take it as further evidence for a direct interaction of Tube and Pelle.

In summary, our results support a model in which Tube mediates signal transduction between Toll and Pelle by direct interaction with Pelle. The phosphorylation target of activated Pelle is still unknown, but candidates are Cactus, Dorsal protein, or another as-yet unidentified protein. Because the active form of Torso, like other receptor tyrosine kinases, is probably a dimer[22], the activating fusion constructs suggest that dimerization may be important for activation of Pelle and Tube. In addition, membrane association may be involved, as fusion with wild-type Torso leads to activation of Pelle and Tube as well, although with lower efficiency. The signalling pathways regulating activation of NF-κB and Dorsal protein might be conserved, as homologous components are involved: Dorsal protein is a member of the rel/NF-κB family[23], Cactus is homologous to I-κB proteins[19,20] and Toll is partially homologous to the interleukin-1 receptor[8,24]. Future research will show whether homologues of Pelle and Tube have similar functions in regulation of NF-κB in vertebrates. □

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