Functional interactions between the pelle kinase, Toll receptor, and tube suggest a mechanism for activation of dorsal

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A complex signal transduction pathway functions in the early *Drosophila* embryo to establish dorsal–ventral polarity. Activation of this pathway results in the nuclear transport of the protein dorsal (dl), a member of the rel/NF- κ B family of transcription factors. Genetic studies have identified three intracellular components whose activity is required for activation of dl: Toll, a transmembrane receptor; pelle (pll), a serine/threonine protein kinase; and tube, a protein of unknown function. Here we examine the activities of these proteins when coexpressed in *Drosophila* Schneider cells. Coexpression of pll with dl enhanced dl nuclear localization and resulted in a modest increase in transcriptional activity. However, when pll was coexpressed with a specific mutant derivative of Toll (TlNaeI), although not with wild-type Toll, a striking synergistic activation of dl was detected. Unexpectedly, coexpression of pll plus TlNaeI, in the absence of dl, resulted in a similar synergistic activation of a GAL4–tube fusion protein. Based on these and other results, we propose a model in which pll receives a signal from activated Toll and phosphorylates tube, which then participates directly in dl activation.

[Key Words: *Drosophila*; signal transduction pathway; rel/NF- κ B proteins; phosphorylation; transcriptional regulation]

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Dorsal–ventral (D/V) polarity is established in the early *Drosophila* embryo by the formation of a nuclear concentration gradient of the maternal morphogen dorsal (dl). This gradient arises from the selective nuclear transport of dl into ventral but not dorsal nuclei (Roth et al. 1989; Rushlow et al. 1989; Steward 1989). dl is a member of the rel/NF- κ B family of transcription factors and, like the other members of this family, its activity is regulated by subcellular localization (for review, see Govind and Steward 1991; Rushlow and Warrior 1992; Norris and Manley 1995a). Genetic screens have identified 11 genes that are required for regulating dl nuclear transport and activity. Seven of these are required for the generation of a signal that is localized in the perivitelline fluid on the ventral side of the embryo (Stein et al. 1991; Stein and Nüsselein-Volhard 1992; Morisato and Anderson 1994; Schneider et al. 1994), whereas the other four are required for transmitting this signal from the plasma membrane to dl. There are several parallels between this intracellular pathway and the signal transduction pathways that regulate NF- κ B. In fact, dl and the closely related protein, Dif (Ip et al. 1993), plus the four intracellular signaling components, are known to function zy-

gotically during the *Drosophila* immune response (Lamaitre et al. 1995).

The *Toll* gene encodes a transmembrane protein that serves in the embryo as the receptor for the ventrally localized signal (Hashimoto et al. 1988, 1991; Morisato and Anderson 1994; Schneider et al. 1994). Toll's intracellular domain is similar to the cytoplasmic domain of the interleukin-1 receptor (IL-1R) (Schneider et al. 1991). Several agents, including the cytokine interleukin-1 (IL-1), lead to the activation of NF- κ B (Shirakawa et al. 1989; for review, see Siebenlist et al. 1995). The similarity between Toll and IL-1R suggests that they may use similar pathways to transmit their signals, although the mechanism of signal transduction is not well understood. However, there is evidence that both may, in some cases, utilize protein kinase A (PKA) to signal nuclear transport, as treatment of cells with IL-1 can raise cAMP levels (Shirakawa et al. 1988) and expression of Toll or PKA activates dl in *Drosophila* Schneider cells (Norris and Manley 1992).

Although the mechanism of signal transduction by Toll is unknown, it is clear that it involves dissociation of dl from its inhibitor, cactus (Roth et al. 1991; Gillespie and Wasserman 1994; Belvin et al. 1995). Activation of NF- κ B also requires dissociation from its inhibitor I κ B, a protein similar to cactus. Phosphorylation of I κ B was

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initially thought to disrupt the I κ B/NF- κ B complex directly because phosphorylation of I κ B in vitro destroyed its ability to inhibit NF- κ B DNA binding (Ghosh and Baltimore 1990). However, in vivo experiments have shown that phosphorylation of I κ B is necessary but not sufficient for activation of NF- κ B (Finco et al. 1994; Aikalay et al. 1995; DiDonato et al. 1995). Increasing evidence suggests that phosphorylation of I κ B regulates its degradation, and the degradation of I κ B appears to result in the disruption of the I κ B/NF- κ B complex (Beg et al. 1993; Henkel et al. 1993; Miyamoto et al. 1994; Palombella et al. 1994; Traenckner et al. 1994, 1995; Brown et al. 1995). cactus is also rapidly degraded in response to activation of the signaling pathway (Belvin et al. 1995), although dl, not cactus, has been shown to be phosphorylated in response to signaling in embryos (Whalen and Steward 1993; Gillespie and Wasserman 1994).

The activities of two genes, *tube* and *pelle* (*p11*), are known from genetic studies to be required downstream of Toll (Hecht and Anderson 1993). The *tube* gene encodes a protein with no homology to any known proteins, whereas *p11* encodes a serine/threonine protein kinase that is most similar to members of the raf/mos family of protein kinases (Letsou et al. 1991; Shelton and Wasserman 1993). *tube* is initially localized to the plasma membrane of the embryo at the time when Toll is activated, although it can be detected subsequently in nuclei (Galindo et al. 1995). Also, *tube* can colocalize with dl to the nucleus in cotransfected Schneider cells (Norris and Manley 1995b). Expression of artificial gain-of-function *tube* and *p11* alleles has shown that *tube* activity can be required upstream of *p11* (Grosshans et al. 1994; Galindo et al. 1995). These results suggest that *tube* may help transmit the signal for dl nuclear transport from Toll to *p11*, resulting in activation of *p11*. The in vivo targets of *p11* are not known, although *p11* phosphorylates *tube*, but not dl or cactus, in vitro (Grosshans et al. 1994).

To investigate further the role of *p11*, as well as Toll and *tube*, in dl signaling we have continued to use transient cotransfection assays in Schneider cells (Norris and Manley 1992, 1995b). *p11*, like Toll and dl but unlike *tube* and cactus, is not expressed endogenously in Schneider cells (J.L. Norris and J.L. Manley, unpubl.). Here we show that expression of *p11* can enhance nuclear localization and transcriptional activity of dl. However, coexpression of *p11* and a specific Toll deletion mutant is shown to result in much stronger, synergistic activation of dl. In addition, *p11* activated by the same Toll derivative markedly increased transcriptional activity of a GAL4-*tube* fusion protein in the absence of dl. Based on these and other findings, we discuss possible models for the interactions among the components of this signal transduction pathway.

Results

Expression of p11 enhances the transcriptional activity of dl

As discussed above, the *p11* gene encodes a protein ki-

nase, and dl is phosphorylated in response to activation of the signal transduction pathway that leads to its nuclear localization. To investigate the function of *p11* in this pathway, we used transient cotransfection assays, as described previously (Norris and Manley 1992, 1995b). To measure dl activity we used a 235-bp fragment from the *Drosophila zen* promoter (−196 to +39) to drive expression of the chloramphenicol acetyl transferase (CAT) gene (*zen*-CAT200; Rushlow et al. 1989). When expressed in Schneider cells, dl has been shown to activate expression from *zen*-CAT200 in a concentration-dependent manner, which reflects, at least in part, an increase in the nuclear concentration of dl (Rushlow et al. 1989; see below). Although the exact mechanism by which dl activates CAT expression from *zen*-CAT200 has not been studied, it likely involves a potential weak dl binding site (see Norris and Manley 1995b).

Figure 1A shows the *p11* construct used in these experiments. The *p11* cDNA was cloned into an actin 5C vector such that the expressed protein contains an amino-terminal influenza virus HA1 epitope tag (ActFlu-*p11*). Figure 1B shows the results of cotransfections that contained 0.4 μ g of Act-dl and the *zen*-CAT200 reporter plasmid, plus or minus ActFlu-*p11*. This concentration of Act-dl was used because, as shown previously, CAT expression is enhanced only weakly unless other genes that can activate dl are cotransfected (Norris and Manley 1992, 1995b). As shown in Figure 1B, expression of *p11* enhanced the ability of dl to activate CAT expression approximately fourfold. This activation was specific to dl, as expression of *p11* in the absence of dl had no effect on expression from *zen*-CAT200 (first line, Fig. 1B), nor did *p11* affect the ability of several other coexpressed transcriptional activators to enhance CAT expression from appropriate reporter plasmids (data not shown).

We also tested the effect of *p11* on the activity of two dl mutants, dl3 and dlQ. The protein encoded by dl3 lacks the carboxy-terminal 117 amino acids and has been shown to be constitutively nuclear in transfected cells (Rushlow et al. 1989). Expression of *p11* enhanced dl3's activation of CAT expression approximately threefold, suggesting that *p11* may induce a modification that affects not only the nuclear localization of dl but also transcriptional activity. The dlQ protein contains glutamine in place of serine at residue 312, the potential PKA phosphorylation site. This protein is localized primarily in the cytoplasm and has only weak transcriptional activity even at high concentrations or in the presence of Toll or PKA (Norris and Manley 1992). Expression of *p11* did not enhance the ability of dlQ to activate CAT expression, suggesting that the PKA site is required for dl nuclear transport or activity.

Expression of p11 enhances dl nuclear localization

When Schneider cells are transfected with 0.4 μ g of Act-dl, dl is located primarily in the cytoplasm (e.g., Rushlow et al. 1989). In contrast, when dl is coexpressed with Toll or PKA, dl is found primarily in the nucleus, or in both the cytoplasm and nucleus in the case of Toll (Norris and

Figure 1. Activity of *dl* in the presence of *pIl*. (A) The *pIl* expression vector containing the HA1 epitope tag (Flu) is shown. The putative amino-terminal regulatory domain and the carboxy-terminal catalytic domain are indicated. Numbers refer to amino acid residues. (B) Schneider cells were cotransfected with 0.4 μ g of *dl* expression vector, 4.0 μ g of *pIl* expression vector, 1.0 μ g of the *zen*-CAT200 reporter plasmid, and pAct5C to bring the final concentration of expression vector to 7.0 μ g. All transfections were normalized for differences in transfection efficiency by using *copia* β -gal as an internal control. Fold activation is presented as the increase in CAT activity relative to the value from the cotransfection lacking a *pIl* expression vector.

Manley 1992). Therefore, part of the enhancement of *dl* activity induced by Toll or PKA can be attributed to the increase in the nuclear concentration of *dl*. Immunofluorescence assays were performed to determine whether the increase in *dl* activity in the presence of *pIl* could also be attributable to an increase in *dl* nuclear concentration. Cells were transfected with either 0.4 μ g of Act-*dl* or 0.4 μ g of Act-*dl* plus 4.0 μ g ActFlu-*pIl*, fixed, and stained with anti-*dl* antibodies. Figure 2A shows that *dl* was localized primarily in the nucleus when *pIl* was expressed. As discussed above, *dlQ* was localized primarily in the cytoplasm even in the presence of Toll or PKA. Because *pIl* could relocalize wild-type (wt) *dl* effectively, we wanted to determine whether *pIl* could also relocalize *dlQ*. Figure 2B shows that expression of *pIl* did not enhance nuclear localization of *dlQ*, suggesting that the PKA site is required for *dl* nuclear localization even when translocation is induced by *pIl*.

We have shown previously that expression of PKA enhanced *dl* transcriptional activity up to 15-fold (Norris and Manley 1992). Because *pIl* and PKA are both kinases that can regulate *dl* subcellular localization we wanted to determine whether the activity of either one might affect the activity of the other. Figure 3 shows the results of cotransfecting Schneider cells with *zen*-CAT200 and Act-*dl*, plus ActFlu-*pIl* and/or Act-PKA. PKA enhanced *dl* activity somewhat better than *pIl*, 10-fold as compared with 4-fold, even though *dl* was localized primarily in the nucleus in the presence of both *pIl* and PKA. The *dl* activity obtained when both kinases were coexpressed was additive, suggesting that neither kinase is responsible for activating the other and that they may have different roles in the signal transduction pathway (see Discussion).

Toll and pIl can functionally interact to enhance dl activity synergistically

Because activated Toll is responsible for transducing the signal that leads to *dl* nuclear transport in embryos, we wanted to determine whether coexpression of Toll, or one of several mutant derivatives, could enhance *pIl* activity. Figure 4A shows the Toll constructs used, which have all been described previously (Norris and Manley 1995b). The Toll^{10b} protein contains a single amino acid change in the extracellular domain that results in a constitutively active protein in embryos (Schneider et al. 1991) and elevated activity in transfected Schneider cells. The TlStuI and TlBstXI constructs contain deletions into the region of the intracellular domain that shares homology with IL-1R and encode inactive proteins. The TlNarI and TlNaeI proteins both maintain the region that is similar to IL-1R but delete part of the region that is unique to Toll. The protein produced by TlNarI retains the same activity as wild-type Toll, whereas the TlNaeI protein was shown to be twofold more active than wild-type Toll. The TlIC construct expresses only the intracellular domain, and the resulting protein was shown to be half as active as full-length Toll. The wild-type and mutant derivatives all accumulated to comparable levels in transfected cells (Norris and Manley 1995b; results not shown).

Figure 4B presents the results of cotransfecting Schneider cells with *zen*-CAT200, Act-*dl*, ActFlu-*pIl*, and each of the above Act-Tl constructs. The *dl* activity detected when Tl, Tl^{10b}, TlNarI, TlStuI, or TlBstXI was coexpressed with *pIl* was additive. However, the *dl* activity observed when TlNaeI was coexpressed with *pIl* was not only much greater than additive, it was signifi-

Figure 2. Subcellular localization of *dl* in the presence of *pll*. Schneider cells were cotransfected with 0.4 μ g of *dl* expression vector alone or with 4 μ g of *pll* expression vector. (A) Cytoplasmic staining of wild-type *dl* alone and nuclear staining of wild-type *dl* in the presence of *pll* is shown. (B) Cytoplasmic staining of *dlQ* both alone and in the presence of *pll* is shown.

cantly greater than what would be expected from multiplying the individual activities. Therefore, *TlNaeI*, but not full-length Toll, can function synergistically with *pll* to enhance *dl* activity. This synergy was specific for *pll* and *TlNaeI*, as cotransfection of Act-PKA and Act-*TlNaeI* had only an additive effect on *dl* activity (results not shown).

A comparison of the Toll and IL-1R intracellular domains is presented in Figure 5A. The two domains share 43% similarity and 26% identity over the entire 212 amino acid residues of the IL-1R intracellular domain and 200 residues of the Toll intracellular domain (Schneider et al. 1991). This region of similarity is re-

quired for the function of both receptors (Heguy et al. 1992; Norris and Manley 1995b). Toll's intracellular domain has an additional 68 residues not found in the IL-1R intracellular domain. It is thus deletion of this Toll-unique region that allowed Toll to interact synergistically with *pll*, extending the view (Norris and Manley 1995b) that this region constitutes an inhibitory domain.

To extend the study of this domain, we also tested the effect of deleting this region in *Toll*^{10b} and *TlIC*. Figure 5B shows the results, presented as Toll + *pll* synergy (see legend to Fig. 5), of cotransfections of each Act-*TlNaeI* deletion with Act-*dl*, ActFlu-*pll* and *zen*-CAT200. In each case, only when the inhibitory domain was deleted was synergistic activation of *dl* detected. This synergy was specific for *dl* as cotransfection of Act-*TlNaeI* and ActFlu-*pll* with Act-*z2* or Act-*prd*, which encode homeo domain activators (Han et al. 1989), did not affect CAT expression from the appropriate reporter plasmids (results not shown). Together, these results indicate that in the absence of the Toll inhibitory domain, Toll and *pll* functionally interact in a manner that results in a dramatic increase in *dl* activity.

dl activity in the presence of *pll* and *tube*

The tube and *pll* proteins have been shown to interact in a yeast two-hybrid system (Grosshans et al. 1994; Gal-

Figure 3. Activity of *dl* in the presence of PKA and *pll*. Schneider cells were transfected with 0.4 μ g of *dl* expression vector, either 3.0 μ g of PKA expression vector or 3.6 μ g of *pll* expression vector alone or in combination, 1.0 μ g of the *zen*-CAT200 reporter plasmid, and pAct5C to bring the concentration of expression vector to 7.0 μ g. The activation values are expressed relative to cotransfections containing the actin 5C expression vector without an insert.

Figure 4. Activity of dl in the presence of Toll and pll. (A) The extracellular and intracellular domains, the region of IL-1R homology, and the inhibitory domain (ID) are indicated on each Toll construct. Numbers refer to amino acid residues. The IC construct contains the HA1 epitope tag (Flu). (B) Schneider cells were cotransfected with 0.4 μ g of dl expression vector, 3.0 μ g of the indicated Toll expression vector, 3.6 μ g of the pll expression vector, 1.0 μ g of the zen-CAT200 reporter plasmid, and pAct5C to bring the concentration of expression vector to 7.0 μ g. The activation values are expressed relative to cotransfections containing 0.4 μ g of the dl expression vector.

indo et al. 1995). To begin to investigate the possible functional significance of this interaction, we cotransfected Schneider cells with zen-CAT200 and Act-dl plus Act-tube and/or ActFlu-pll, and the results are presented in Figure 6A. Under these conditions, dl activated CAT expression ~20-fold in the presence of pll and 15-fold in the presence of tube. However, when pll and tube were coexpressed dl activated CAT expression by only

~10-fold, indicating that expression of pll and tube together not only did not enhance dl activation but had a slight inhibitory effect. To determine whether activation of the signaling pathway by TlNael could prevent this inhibition, Act-TlNael was included in cotransfections. Figure 6B shows that when TlNael was coexpressed there was no inhibition of the ability of tube or pll to activate dl. However, tube was unable to enhance the

Figure 5. Activity of dl in the presence of dl and Toll inhibitory domain deletions. (A) The intracytoplasmic domains of IL-1R and Toll are shown. Regions with >50% similarity are shaded. Numbers refer to amino acid residues. (B) Schneider cells were cotransfected with 0.4 μ g of dl expression vector, 3.6 μ g of pll expression vector, either 3.0 μ g of wild-type Tl, TlNael, TlIC, TlICNael or 1.0 μ g of Tl10b, Tl10bNael, 1.0 μ g of the zen-CAT200 reporter plasmid, and pAct5C to bring the final concentration of expression vector to 7.0 μ g. The values represent how many times higher the observed value of dl activity in the presence of the indicated Toll derivative plus pll is relative to the value that would be obtained by multiplying the dl activities detected in the presence of either the Toll derivative or pll alone. Thus, a value of 1.0 or higher is equal to or greater than multiplicative.

Figure 6. Activity of *dl* in the presence of *tube* and *pll*. (A) Schneider cells were cotransfected with 0.4 μ g of *dl* expression vector, 3.0 μ g of *pll* expression vector, 3.0 μ g of *tube* expression vector, 1.0 μ g of the *zen*-CAT200 reporter plasmid, and pAct5C to bring the concentration of expression vector to 7.0 μ g. The activation values are expressed relative to cotransfections containing the actin 5C expression vector without an insert. (B) Schneider cells were transfected with 0.4 μ g of *dl* expression vector, 2.0 μ g of *pll* expression vector, 2.0 μ g of *tube* expression vector, 2.0 μ g of *TlNaeI* expression vector, 1.0 μ g of the *zen*-CAT200 reporter plasmid, and pAct5C to bring the concentration of expression vector to 7.0 μ g.

activation brought about by *TlNaeI* plus *pll*, which may reflect the fact that *tube* is present endogenously in Schneider cells (see Norris and Manley 1995b).

pll can enhance the activity of a GAL4-*tube* fusion protein

As mentioned above, *pll* has been shown to phosphorylate *tube* but not *dl* in vitro (Grosshans et al. 1994). In addition, we showed that *tube* can colocalize with *dl* to the nucleus, where it has weak transcriptional activating potential. This led us to suggest that *tube* may function, at least in part, as a coactivator of *dl*. These properties of *tube* raise the possibility that it, rather than *dl*, could be the target of *TlNaeI*-activated *pll*. To test this we utilized the ActGAL4-*tube* expression vector we described previously. This construct consists of the *tube* coding region fused to a fragment encoding the GAL4 DNA-binding domain, and the resulting fusion protein was shown to activate transcription from a promoter containing GAL4 binding sites three- to fourfold (Norris and Manley 1995b).

ActGAL4-*tube* was cotransfected into Schneider cells with ActFlu-*pll*, Act-*TlNaeI*, or the two together. All cotransfections contained a reporter plasmid with five GAL4 binding sites controlling expression to the *CAT* gene (G5E1bTATA-CAT; Colgan et al. 1993), and the results are shown in Figure 7A. Expression of either *pll* or *TlNaeI* alone had no significant effect on the activity of GAL4-*tube*. However, expression of *pll* plus *TlNaeI* resulted in a striking 70-fold activation of *CAT* expression

by GAL4-*tube*. This effect was specific for GAL4-*tube*, as expression of *pll* and *TlNaeI* did not enhance the activity of either GAL4-VP16 (Fig. 7B) or a construct containing only the GAL4 DNA-binding domain (results not shown). Activation was also completely dependent on the presence of GAL4 sites in the reporter plasmid (results not shown). To determine whether this effect required a functional *tube* protein we used a GAL4-*tube2* fusion construct. The *tube2* protein has a single amino acid change of glutamate to lysine at residue 140, which inactivates the protein in embryos (Letsou et al. 1993) and in transfected cells (Norris and Manley 1995b). Figure 7C shows that no activation of *CAT* expression was observed when ActGAL4-*tube2* was cotransfected with ActFlu-*pll* and Act-*TlNaeI*. Although we have not measured accumulation of the GAL4-*tube* proteins, expression of *tube* and *tube2* in Schneider cells is comparable (Norris and Manley 1995b).

pll kinase activity is required for signaling

To determine whether the effects of *pll* expression we observed reflected phosphorylation, a *pll* mutant that lacks kinase activity was tested. When the lysine residue at position 240 in the catalytic domain is changed to arginine (K240R), a mutation predicted to abolish enzymatic activity, the resulting protein could not rescue *pll* null embryos (Shelton and Wasserman 1993). A *pll* cDNA containing this mutation was cloned into the actin 5C expression vector, Act-*pll*K240R, which was cotransfected with Act-*dl*, Act-*dl* plus Act-*TlNaeI* (Fig.

Figure 7. Activity of GAL4-tube fusion proteins in the presence of pll and Toll. Schneider cells were cotransfected with either 3.0 μ g of GAL4-tube (A) or 1.0 ng GAL4-VP16 (B) or 3.0 μ g GAL4-tube2 (C) expression vector, 3.0 μ g of pll expression vector, 1.0 μ g of TlNaeI expression vector, 1.0 μ g of the G5E1bTATA-CAT reporter plasmid, and pAct5C to bring the concentration of expression vector to 7.0 μ g. Activation values are expressed relative to cotransfections containing the actin 5C expression vector without an insert. (B) As a control to ensure that a lack of response was not attributable to a factor limiting for expression in the presence of the strong activator GAL4-VP16, cells were also transfected with 5.0 ng of the GAL4-VP16 expression vector.

8A), or ActGAL4-tube plus Act-TlNaeI (Fig. 8B). The results show that pllK240R did not, by itself, enhance dl activity, nor did it synergistically activate dl or GAL4-tube in the presence of TlNaeI. In fact, a reduction in both dl and GAL4-tube activity was observed when pllK240R and TlNaeI were coexpressed, which likely reflects a dominant-negative effect of the mutant kinase (see Discussion). To determine whether pllK240R could relocate dl to the nucleus, immunofluorescence assays were performed. Figure 8C shows that dl remained localized primarily in the cytoplasm in the presence of pllK240R. Therefore, the K240R mutation abolishes the ability of pll to enhance both nuclear localization and transcriptional activity.

Discussion

Models for activation of dl nuclear transport and transcriptional activity

The data presented here provide new insights into how

components of the dl signaling pathway can interact with one another during signal transduction. These interactions, as well as others suggested by previous studies, are summarized schematically in Figure 9. Prior to ligand binding (Fig. 9, top), we suggest that Toll's intracellular domain is in a conformation where the active domain, the region that is similar to IL-1R, is masked by the inhibitory domain, the region unique to Toll. The tube and pll proteins are complexed (Grosshans et al. 1994; Galindo et al. 1995) and localized near the plasma membrane, whereas the dl and cactus proteins are complexed (Geisler et al. 1992; Kidd 1992) in the cytoplasm. Once Toll is activated a conformational change occurs in the intracellular domain resulting in exposure of the active domain.

The intracellular interactions that follow Toll activation are still not clear, although several possible mechanisms of signal transmission from Toll to dl are possible. In a previous study (Norris and Manley 1992), we presented evidence that Toll can activate an intracellular

Figure 8. Activity of *dl* and GAL4-*tube* in the presence of *pllK240R* and *TlNaeI*. (A) Schneider cells were cotransfected with 0.4 μ g of *dl* expression vector, 3.0 μ g of *pll* or *pllK240R* expression vector, 1.0 μ g of *TlNaeI* expression vector, 1.0 μ g of the *zen*-CAT200 reporter plasmid, and pAct5C to bring the final concentration of expression vector to 7.0 μ g. (B) Schneider cells were cotransfected with 3.0 μ g of GAL4-*tube* expression vector, 3.0 μ g of *pll* or *pllK240R* expression vector, 1.0 μ g of *TlNaeI* expression vector, 1.0 μ g of the G5E1bTATA-CAT reporter plasmid, and pAct5C to bring the concentration of expression vector to 7.0 μ g. All activation values (A,B) are expressed relative to cotransfections containing the actin 5C expression vector without an insert. (C) Schneider cells were cotransfected with 0.4 μ g of *dl* expression vector alone or with 4 μ g of *pllK240R* expression vector. Cells were fixed and stained with anti-*dl* and TRITC-conjugated antibodies.

signaling pathway in Schneider cells that utilizes PKA to enhance *dl* nuclear localization and transcriptional activity (Fig. 9, bottom left; see also below). Recent genetic studies (Grosshans et al. 1994; Galindo et al. 1995) have suggested that Toll activates a signaling pathway that utilizes *pll* to enhance *dl* nuclear transport. These studies also provided evidence that *tube* activity is required upstream of *pll*, perhaps to facilitate transmission of the signal from Toll to *pll* (Fig. 9, bottom center). Finally, the results presented here also suggest that Toll activates *pll*. However, *pll* then phosphorylates and activates *tube*, which translocates to the nucleus with *dl*, and *tube* thus has a function downstream of *pll* (Fig. 9, bottom right). Because *tube* is expressed endogenously in Schneider cells, its activity may also be required upstream of *pll*.

It is conceivable that the above mechanisms may reflect different aspects of a single pathway. By this view, we would envision an integrated model by which the *tube/pll* complex interacts with the Toll active domain, resulting in activation of *pll*. *pll* then phosphorylates *tube*, which causes the disruption of the *tube/pll*/Toll complex. *tube* is then free to translocate to the nucleus with *dl* (following its dissociation from *cactus*), *pll* is free to modify other proteins (e.g., *dl* or *cactus*), and Toll is free in some way to signal the activation of PKA. Activated PKA phosphorylates *dl*, aiding in the dissociation of the *dl/cactus* complex, or in *dl* nuclear transport, and enhancing *dl* transcriptional activity. However, it is also possible that the three pathways depicted in Figure 9

reflect distinct mechanisms that occur under different circumstances. For example Toll, *pll*, *tube*, and *dl* are known to function not only in the early embryo during D/V patterning but also in larval and adult fat bodies during the immune response (Lemaitre et al. 1995). As with *rel/NF- κ B* proteins in mammals, there may be multiple ways to activate such proteins in *Drosophila*.

The *tube/pll*/Toll complex: transmission of the signal

We have presented results indicating that an interaction, either direct or indirect, occurs between *pll* and Toll upon exposure of Toll's active domain by deletion of the inhibitory domain. We suggest that naturally a ligand-induced conformational change in Toll similarly exposes the active domain. In our system, where Toll is activated in the absence of ligand [presumably by overexpression and aggregation (see Norris and Manley 1995b)], this conformational change may not occur efficiently, and full activation thus requires deletion of the inhibitory domain. This type of effect has also been observed with the Fas receptor and its associated protein FADD. In this case, deletion of 15 carboxy-terminal amino acids from Fas was shown to result in a stronger interaction with FADD in vitro (Chinnaiyan et al. 1995).

Several lines of evidence support the interaction of *pll* with other components of the signaling pathway. Genetic analysis of *pll* alleles has suggested that *pll* may participate in a multicomponent complex. Unlike most

Figure 9. Models for nuclear transport and activation of *dl*. Before activation (*top*), the Toll active domain is masked by its inhibitory domain (solid box), tube and pll are complexed at the plasma membrane, and *dl* is held in the cytoplasm by association with cactus. After Toll is activated, the intracellular active domain is exposed, resulting in the activation of intracellular signaling pathways (*bottom*). These pathways may utilize PKA (*left*), pll (*center and right*), or both to enhance *dl* nuclear transport and activity. tube may function upstream of pll (*center*), downstream of pll (*right*) or at both positions in the pathway (see text). Open arrowheads indicate phosphorylations.

of the other *dl* group genes, several *pll* alleles are cold sensitive, which is often interpreted as suggesting participation of the protein in a complex (Hecht and Anderson 1993). In addition, several *pll* alleles are antimorphic, producing a stronger dorsalized phenotype when expressed with a weak *pll* allele than when the same *pll* allele is expressed with a *pll* deficiency (Hecht and Anderson 1993). Such alleles may interfere with the activity of the signaling pathway by directly and nonproductively interacting with another component. Our results with the pllK240R mutant also provide support for interaction of pll with other components of the signaling pathway. If pll participates in a complex with Toll, and its kinase activity is required for disrupting this complex, then signal transduction would be blocked if the Toll/pll complex could not be disrupted. In this case, *dl* activity in the presence of pllK240R and Toll should be lower than *dl* activity in the presence of Toll alone because no signaling from Toll could take place. Consistent with this, *dl*, or GAL4–tube, activity in the presence of pllK240R and activated Toll (TlNaeI) was lower than in the presence of activated Toll alone. This reduction in

dl activity could result, for example, from an inability of Toll to activate PKA, and therefore *dl*, as Toll would remain complexed with pllK240R. In the case of GAL4–tube, pllK240R would be unable to phosphorylate tube, and therefore GAL4–tube would remain complexed with pllK240R and Toll.

Previous studies suggested that tube has another function in regulating *dl*: tube may function as a transcriptional coactivator, as it colocalizes with *dl* in the nucleus and a GAL4–tube fusion protein can activate transcription (Norris and Manley 1995b). Because pll phosphorylates tube in vitro (Grosshans et al. 1994), phosphorylation could provide a mechanism for activation of tube. Because tube is expressed endogenously in Schneider cells, activation of *dl* by pll could function, at least in part, through pll’s activation of tube. Phosphorylation of tube could first provide the mechanism for the dissociation of the tube/pll/Toll complex and then enhance its nuclear transport, transcriptional activity, or both. The striking activation of GAL4–tube by activated pll supports this model. These results raise the possibility that, perhaps for a limited number of target genes, a significant function of *dl* may be to recruit phosphorylated tube to the promoter.

The *dl*/cactus complex: Activation of *dl*

Our previous work has suggested that phosphorylation of *dl* by PKA can facilitate *dl* nuclear transport and enhance its transcriptional activity (Norris and Manley 1992). PKA appears to function through the conserved PKA site in *dl*’s rel homology domain (RHD), as mutation of this site resulted in a primarily cytoplasmic protein (*dl*Q) that was unresponsive to PKA (or Toll) expression (Norris and Manley 1992). Somewhat unexpectedly, this site also appears to be required for pll-induced *dl* nuclear transport, as expression of pll could not enhance nuclear localization of *dl*Q. We suggest several possible explanations for this finding. First, it is conceivable that pll and PKA recognize the same or related sequences such that the same serine would be modified by each. Second, pll could phosphorylate and activate an endogenous PKA-like kinase, which would then phosphorylate *dl*. Third, the *dl*Q mutation could have an unexpected effect on *dl* not related to pll phosphorylation.

Although in mammalian systems attention has recently centered on I κ B, phosphorylation of NF- κ B may also be involved in regulating NF- κ B/I κ B complex dissociation and/or NF- κ B activity. The p65 subunit of NF- κ B is phosphorylated with the same kinetics as I κ B upon cytokine stimulation of HeLa cells, suggesting that both I κ B and NF- κ B have to be phosphorylated for complex dissociation (Naumann and Scheidereit 1994). Phosphorylation of p65 appears to play a subsequent role in regulation of NF- κ B activity (Naumann and Scheidereit 1994; Neumann et al. 1995). Phosphorylated p65 isolated from HeLa cells, or phosphorylated in vitro (by PKA), exhibits enhanced DNA-binding activity relative to unphosphorylated p65, suggesting that phosphorylation, perhaps by

PKA, is important for optimal activity of p65 (Naumann and Scheidereit 1994).

The results presented here suggest that signal transduction from Toll occurs through pll and tube. The interaction between activated Toll and pll may be direct or may require other components of the signal transduction pathway that have (e.g., tube) or have not been identified. Examination of factors that can directly interact with Toll's intracellular domain, specifically the IL-IR homology region, should clarify the nature of this interaction. Our data have shown that signaling from pll can occur, at least in part, via phosphorylation of tube. Although identification of other proteins that pll modifies may be required to clarify the full role of pll in enhancing dl nuclear transport and activity, our results have provided mechanistic insights into this genetically well-defined signal transduction pathway.

Materials and methods

Recombinant plasmids

All expression vectors were derived from a plasmid that contains the *Drosophila* actin 5C promoter and poly(A) site, pAct5C, which has been described in detail (Han et al. 1989). The ActFlu-pll and Act-pllK240R expression vectors were constructed from the pll cDNA clones kindly provided by S. Wasserman (Shelton and Wasserman 1993). Constructs both with and without an influenza virus HA1 epitope tag were made by standard cloning procedures. The actin 5C expression vector containing the HA1 epitope tag was described previously (pActPflu, Han and Manley 1993). The GAL4-VP16 expression vector was provided by J. Colgan (Colgan et al. 1993). All other expression vectors and reporter constructs used in these studies have been described previously (Han et al. 1989; Rushlow et al. 1989; Norris and Manley 1992, 1995b).

DNA transfection and transient expression assay

Drosophila Schneider L2 cells were grown and transfected as described previously (Han et al. 1989). All transfections contained the indicated amount of each expression vector and variable amounts of pAct5C to bring the total amount of expression vector to 7.0 μ g. All transfections for measuring CAT activities also contained 1.0 μ g of the zen-CAT200 reporter plasmid and 2.0 μ g of the copia long terminal repeat (LTR)-lacZ plasmid (copia- β gal) as an internal control. All transfections were performed in duplicate, and β -galactosidase and CAT activities were measured as described previously (Han et al. 1989). Normalized CAT activities were calculated by determining CAT/ β -gal activity ratios and averaging the values from several independent transfections.

Staining of cells

Cell staining was performed essentially as described previously (Norris and Manley 1992). Briefly, cells were fixed and incubated with anti-dl primary antibodies (Norris and Manley 1995b). Cells were then washed three times, and dl protein was visualized by incubation with the anti-rat TRITC-conjugated secondary antibodies.

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Functional interactions between the pelle kinase, Toll receptor, and tube suggest a mechanism for activation of dorsal.

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