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-κB 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 (TINael), although not with wild-type Toll, a striking synergistic activation of dl was detected. Unexpectedly, coexpression of pll plus TINael, 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-κB 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-κB 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-κB. In fact, dl and the closely related protein, Dif (lp et al. 1998), plus the four intracellular signaling components, are known to function zygotically 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-κB (Shirakawa et al. 1988, 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-κB also requires dissociation from its inhibitor IkB, a protein similar to cactus. Phosphorylation of IkB was
Expression of 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. As discussed above, the pll gene encodes a protein kinase that is activated in response to signaling in embryos (Whalen and Wasserman 1993; Gillespie and Wasserman 1994).

The activities of two genes, tube and pelle (plib), 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 plb encodes a serine/threonine protein kinase that is most similar to members of the Raf/mos family of protein kinases (Letson 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 plb alleles has shown that tube activity can be required upstream of plb (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 plb, resulting in activation of plb. The in vivo targets of plb are not known, although plb phosphorylates tube, but not dl or cactus, in vitro (Grosshans et al. 1994).

To investigate further the role of plb, 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). pll, 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 pll can enhance nuclear localization of dl and activate CAT expression approximately threefold, with pll affecting 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 pll 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 pll enhanced dl3's activation of CAT expression approximately threefold, suggesting that pll 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 pll 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 pll enhances dl nuclear localization

When Schneider cells are transfected with 0.4 {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...
Norris et al.

Figure 1. Activity of dl in the presence of pll. (A) The pll 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 μg of dl expression vector, 4.0 μg of pll expression vector, 1.0 μg of the zen-CAT200 reporter plasmid, and pAct5C to bring the final concentration of expression vector to 7.0 μg. 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 pll expression vector.

We have shown previously that expression of PKA enhanced dl transcriptional activity up to 15-fold (Norris and 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 pll could also be attributable to an increase in dl nuclear concentration. Cells were transfected with either 0.4 μg of Act-dl or 0.4 μg of Act-dl plus 4.0 μg ActFlu-لل, fixed, and stained with anti-dl antibodies. Figure 2A shows that dl was localized primarily in the nucleus when pll was expressed. As discussed above, dlQ was localized primarily in the cytoplasm even in the presence of Toll or PKA. Because pll could relocalize wild-type (wt) dl effectively, we wanted to determine whether pll could also relocalize dlQ. Figure 2B shows that expression of pll did not enhance nuclear localization of dlQ, suggesting that the PKA site is required for dl nuclear localization even when translocation is induced by pll.

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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 pll activity. Figure 4A shows the Toll constructs used, which have all been described previously (Norris and Manley 1995b). The Toll10b 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 T1Stul and T1BstXI constructs contain deletions into the region of the intracellular domain that shares homology with IL-1R and encode inactive proteins. The T1NarI and T1NaeI 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 T1NaeI retains the same activity as wild-type Toll, whereas the T1NaeI protein was shown to be twofold more active than wild-type Toll. The T11C 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-لل, and each of the above Act-Тl constructs. The dl activity detected when Тl, Тl10b, ТlNarI, ТlStul, or T1BstXI was coexpressed with pll was additive. However, the dl activity observed when T1NarI was coexpressed with pll was not only much greater than additive, it was signifi-
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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

and Toll

Figure 5B shows the results, presented as Toll + pll synergy (see legend to Fig. 5), of cotransfections of each Act–TlNael 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–TlNael 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-

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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 TlNaeI could prevent this inhibition, Act–TlNaeI was included in cotransfections. Figure 6B shows that when TlNaeI was coexpressed there was no inhibition of the ability of tube or pll to activate dl. However, tube was unable to enhance the

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 μg of dl expression vector, 3.0 μg of the indicated Toll expression vector, 3.6 μg of the pll expression vector, 1.0 μg of the zen–CAT200 reporter plasmid, and pAct5C to bring the concentration of expression vector to 7.0 μg. The activation values are expressed relative to cotransfections containing 0.4 μg of the dl expression vector.

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 μg of dl expression vector, 3.6 μg of pll expression vector, either 3.0 μg of wild-type Tl, TlNaeI, TlIC, TlICNaeI or 1.0 μg of Tl10b, Tl10bNaeI, 1.0 μg of the zen–CAT200 reporter plasmid, and pAct5C to bring the final concentration of expression vector to 7.0 μg. 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.
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Figure 6. Activity of dl in the presence of tube and pll. (A) Schneider cells were cotransfected with 0.4 μg of dl expression vector, 3.0 μg of pll expression vector, 3.0 μg of tube expression vector, 1.0 μg of the zen−CAT200 reporter plasmid, and pAct5C to bring the concentration of expression vector to 7.0 μg. 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 μg of dl expression vector, 2.0 μg of pll expression vector, 2.0 μg of tube expression vector, 2.0 μg of T1NaeI expression vector, 1.0 μg of the zen−CAT200 reporter plasmid, and pAct5C to bring the concentration of expression vector to 7.0 μg.

Activation brought about by T1NaeI 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 T1NaeI-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–T1NaeI, or the two together. All cotransfections contained a reporter plasmid with five GAL4 binding sites controlling expression to the CAT gene (G5ElbTATA–CAT; Colgan et al. 1993), and the results are shown in Figure 7A. Expression of either pll or T1NaeI alone had no significant effect on the activity of GAL4–tube. However, expression of pll plus T1NaeI 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 T1NaeI 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–T1NaeI. 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–pllK240R, which was cotransfected with Act–dl, Act–dl plus Act–T1NaeI [Fig.
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...
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Figure 8. Activity of dl and GAL4–tube in the presence of pllK240R and T1NaeI. (A) Schneider cells were cotransfected with 0.4 μg of dl expression vector, 3.0 μg of pll or pllK240R expression vector, 1.0 μg of T1NaeI expression vector, 1.0 μg of the zen–CAT200 reporter plasmid, and pAct5C to bring the final concentration of expression vector to 7.0 μg. (B) Schneider cells were cotransfected with 3.0 μg of GAL4–tube expression vector, 3.0 μg of pll or pllK240R expression vector, 1.0 μg of T1NaeI expression vector, 1.0 μg of the G5ElbTATA–CAT reporter plasmid, and pAct5C to bring the concentration of expression vector to 7.0 μg. 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 μg of dl expression vector alone or with 4 μg 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-xB 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
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 \( Toll \) [TINaell] 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.
PKA, is important for optimal activity of p65 [Naumann and Scheidereit 1994].

The results presented here suggest that signal transduction from Toll occurs through pII and tube. The interaction between activated Toll and pl1 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 pl1 can occur, at least in part, via phosphorylation of tube. Although identification of other proteins that pl1 modifies may be required to clarify the full role of pl1 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 ActFluc-pII and Act-pIIK240R expression vectors were constructed from the pII 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 [Han et al. 1989]. 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 ng. All transfections for measuring CAT activities also contained 1.0 μg of the zen-CAT200 reporter plasmid and 2.0 ng of the copia long terminal repeat (LTR)-lacZ plasmid [copia–βgal] 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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References

Aikalay, I., A. Yaron, A. Hatzubai, S. Jung, A. Avraham, O. Gerlitz, I. Pashut-Lavon, and Y. Ben-Neriah. 1995. In vivo stimulation of IκB phosphorylation is not sufficient to activate NF-κB. Mol. Cell. Biol. 15: 1294–1301.

Beg, A.A. and A.S. Baldwin, Jr. 1993. The IκB proteins: Multifunctional regulators of Rel/NF-κB transcription factors. Genes & Dev. 7: 2064–2070.

Beg, A.A., T.S. Finco, P.V. Nantermet, and A.S. Baldwin, Jr. 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκB: A mechanism for NF-κB activation. Mol. Cell. Biol. 13: 3301–3310.

Belvin, M.P., Y. Jin, and K.V. Anderson. 1995. Cactus protein degradation mediates Drosophila dorsal–ventral signaling. Genes & Dev. 9: 783–793.

Brown, K., S. Gerstberger, L. Carlson, G. Franzoso, and U. Sieweck, B. 1995. Control of IκB-α proteolysis by site-specific signal-induced phosphorylation. Science 267: 1485–1488.

Chinnaiyan, A.M., K. O’Rourke, A. Tewari, and V.M. Dixit. 1993. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 81: 505–512.

Colgan, J., S. Wampler, and J.L. Manley. 1993. Interaction between a transcriptional activator and transcription factor IIB in vivo. Nature 362: 549–553.

DiDonato, J.A., F. Mercurio, and M. Karin. 1995. Phosphorylation of IκBα precedes but is not sufficient for its dissociation from NF-κB. Mol. Cell. Biol. 15: 1302–1311.

Finco, T.S., A.A. Beg, and A.S. Baldwin, Jr. 1994. Inducible phosphorylation of IκBα is not sufficient for its dissociation from NF-κB and is inhibited by protease inhibitors. Proc. Natl. Acad. Sci. 91: 11884–11888.

Galindo, R.L., D.N. Edwards, S.K.H. Gillespie, and S.A. Wasserman. 1995. Interception of the pelle kinase with the membrane-associated protein tube is required for transcription of the dorsal-ventral signal in Drosophila embryos. Development 121: 2209–2218.

Geisler, R., A. Bergmann, Y. Hiromi, and C. Nüsslein-Volhard. 1992. cactus, a gene involved in dorsal-ventral pattern formation of Drosophila, is related to the IκB gene family of vertebrates. Cell 71: 613–621.

Ghosh, S. and D. Baltimore. 1990. Activation in vitro of NfκB by phosphorylation of its inhibitor IκB. Nature 344: 678–682.

Gillespie, S.K. and S.A. Wasserman. 1994. dorsal, a Drosophila Rel-like protein, is phosphorylated upon activation of the transmembrane protein Toll. Mol. Cell. Biol. 14: 3559–3568.

Govind, S. and R. Steward. 1991. Dorsal-ventral pattern formation in Drosophila: Signal transduction and nuclear targeting. Trends Genet. 7: 119–125.

Grosshans, J., A. Bergmann, P. Haffter, and C. Nüsslein-Volhard. 1994. Activation of the kinase Pelle by Tube in the dorsal-ventral signal transduction pathway of Drosophila embryo. Nature 372: 563–566.

Han, K. and J.L. Manley. 1993. Transcriptional repression by the Drosophila Even-skipped protein: Definition of a minimal repression domain. Genes & Dev. 7: 491–503.
Norris et al.

Han, K., M.S. Levine, and J.L. Manley. 1989. Synergistic activation and repression of transcription by Drosophila homeobox proteins. Cell 56: 573–583.

Hashimoto, C., K.L. Hudson, and K.V. Anderson. 1988. The Toll gene of Drosophila, required for dorsal–ventral embryonic polarity, appears to encode a transmembrane protein. Cell 52: 269–279.

Hashimoto, C., S. Gertruda, and K.V. Anderson. 1991. Plasma membrane localization of the Toll protein in the syncytial Drosophila embryo: Importance of transmembrane signaling for dorsal–ventral pattern formation. Development 111: 1020–1028.

Hecht, P.M. and K.V. Anderson. 1993. Genetic characterization of tube and pelle, genes required for signaling between Toll and dorsal in the specification of the dorsal-ventral pattern of the Drosophila embryo. Genetics 135: 405–417.

Heguy, A., C.T. Baldari, G. Macchia, J.L. Telford, and M. Melli. 1992. Amino acids conserved in interleukin-1 receptors (IL-1Rs) and the Drosophila Toll protein are essential for IL-1R signal transduction. J. Biol. Chem. 267: 2605–2609.

Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Nerish, and P.A. Baeuerle. 1993. Rapid proteolysis of IkBα is necessary for activation of transcription factor NFkB. Nature 365: 182–185.

Ip, Y.T., M. Reach, Y. Engstrom, L. Kadalayil, H. Cai, S. Gonzalez-Crespo, K. Tatei, and M. Levine. 1993. Dif, a dorsal-related regulatory gene that mediates an immune response in Drosophila. Cell 75: 753–765.

Kidd, S. 1992. Characterization of the Drosophila cactus locus and analysis of interactions between cactus and dorsal proteins. Cell 71: 623–635.

Lamaitre, B., M. Meister, S. Govind, P. Georgel, J.M. Reichart, and J. Hoffmann. 1995. Functional analysis and regulation of nuclear import of dorsal during the immune response in Drosophila. EMBO J. 14: 536–545.

Letsou, A., S. Alexander, and S.A. Wasserman. 1991. Genetic and molecular characterization of tube, a Drosophila gene maternally required for embryonic dorsoventral polarity. Proc. Natl. Acad. Sci. 88: 810–814.

Letsou, A., S. Alexander, and S.A. Wasserman. 1993. Domain mapping of tube, a protein essential for dorsoventral patterning of the Drosophila embryo. EMBO J. 12: 3449–3458.

Miyamoto, S., M. Maki, M.J. Schmitt, M. Hatanaka, and I.M. Verma. 1994. Tumor necrosis factor α-induced phosphorylation of IkBα is a signal for its degradation but not dissociation from NFkB. Proc. Natl. Acad. Sci. 91: 12740–12744.

Morisato, D. and K.V. Anderson. 1994. The spätzle gene encodes a component of the extracellular signaling pathway establishing the dorsal-ventral pattern of the Drosophila embryo. Cell 76: 677–688.

Naumann, M. and C. Scheiderer. 1994. Activation of NF-κB in vivo is regulated by multiple phosphorylations. EMBO J. 13: 4597–4607.

Neumann, M., T. Grieshammer, S. Chuvpilo, B. Knue, and B. Ernfsson, Jr., and J. Eserling. 1985. RelA/p65 is a cellular protein that regulates the NF-kappa B-like transcription factor in unstimulated cells. EMBO J. 14: 1991–2004.

Norris, J.L. and J.L. Manley. 1992. Selective nuclear transport of the Drosophila morphogen dorsal can be established by a signaling pathway involving the transmembrane protein Toll and the protein kinase A. Genes & Dev. 6: 1654–1667.

———. 1995a. Regulation of the nuclear transport and activity of the Drosophila morphogen Dorsal. In Inducible gene expression, Volume 2 (ed. P.A. Baeuerle), pp. 243–265. Birkhäuser, Boston, MA.

———. 1995b. Regulation of dorsal in cultured cells by Toll and tube: tube function involves a novel mechanism. Genes & Dev. 9: 358–369.

Palombella, V.J., O.J. Rando, A.L. Goldberg, and T. Maniatis. 1994. The ubiquitin-proteasome pathway is required for processing the NF-κB precursor protein and the activation of NF-κB. Cell 78: 773–785.

Roth, S., D. Stein, and C. Nüsslein-Volhard. 1989. A gradient of nuclear localization of the dorsal protein determines dorsoventral pattern in the Drosophila embryo. Cell 59: 1165–1177.

Roth, S., Y. Hiromi, D. Godt, and C. Nüsslein-Volhard. 1991. Cactus, a maternal gene required for proper formation of the dorsoventral morphogen gradient in Drosophila embryos. Development 112: 371–388.

Rushlow, C. and R. Warrior. 1992. The Rel family of proteins. BioEssays 14: 89–95.

Rushlow, C., K. Han, J.L. Manley, and M. Levine. 1989. The graded distribution of the dorsal morphogen is initiated by selective nuclear transport in Drosophila. Cell 59: 1165–1177.

Schneider, D.S., K.L. Hudson, T.Y. Lin, and K.V. Anderson. 1991. Dominant and recessive mutations define functional domains of Toll, a transmembrane protein required for dorsal–ventral polarity in the Drosophila embryo. Genes & Dev. 5: 797–807.

Schneider, D.S., S.Y. Yin, D. Morisato, and K.V. Anderson. 1994. A processed form of the spätzle protein defines dorsal-ventral polarity in the Drosophila embryo. Development 120: 1243–1250.

Shelton, C.A. and S.A. Wasserman. 1993. pelle encodes a protein kinase required to establish dorsoventral polarity in the Drosophila embryo. Cell 72: 515–525.

Shirakawa, F., U. Yamashita, M. Chedid, and S.B. Mizel. 1988. Cyclic AMP—an intracellular second messenger for interleukin 1. Proc. Natl. Acad. Sci. 85: 8201–8205.

Shirakawa, F., M. Chedid, J. Suttles, B.A. Pollok, and S.B. Mizel. 1989. Interleukin 1 and cyclic AMP induce κ immunoglobulin light-chain expression via activation of an NF-κB-like DNA-binding protein. Mol. Cell. Biol. 9: 959–964.

Sibben, U., K. Brown, and G. Franzoso. 1995. NF-κB: A mediator of pathogen and stress responses. In Inducible gene expression, Volume 1 (ed. P.A. Baeuerle), pp. 93–141. Birkhäuser, Boston, MA.

Stein, D. and C. Nüsslein-Volhard. 1992. Multiple extracellular activities in Drosophila egg perivitelline fluid are required for establishment of embryonic dorsal–ventral polarity. Cell 68: 429–440.

Stein, D., S. Roth, E. Vogelsang, and C. Nüsslein-Volhard. 1991. The polarity of the dorsoventral axis in the Drosophila embryo is defined by an extracellular signal. Cell 65: 725–735.

Steward, R. 1989. Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. Cell 59: 1179–1188.

Traenckner, E.B.-M., S. Wilk, and P.A. Baeuerle. 1994. A proteasome inhibitor prevents activation of NF-κB and stabilizes a newly phosphorylated form of IkBα that is still bound to NF-κB. EMBO J. 13: 5433–5441.

Traenckner, E.B.-M., H.L. Pahl, T. Henkel, K.N. Schmidt, S. Wilk, and P.A. Baeuerle. 1995. Phosphorylation of human IkBα on serines 32 and 36 controls IkBα proteolysis and NF-κB activation in response to diverse stimuli. EMBO J. 14: 2876–2883.

Whalen, A.M. and R. Steward. 1993. Dissociation of the Dorsal–Cactus complex and phosphorylation of the Dorsal protein correlate with the nuclear localization of Dorsal. J. Cell Biol. 123: 523–534.
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