MOLECULAR AND CELLULAR BIOLOGY, June 1994, p. 3559–3568
0270-7306/94/$04.00+0

Vol. 14, No. 6

# dorsal, a *Drosophila* Rel-Like Protein, Is Phosphorylated upon Activation of the Transmembrane Protein Toll

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Received 11 November 1993/Returned for modification 22 December 1993/Accepted 31 January 1994

**The nuclear import of dorsal, a *Drosophila* Rel homolog, is directed by a spatially restricted extracellular ligand in blastoderm embryos. We have demonstrated both that dorsal is an embryonic phosphoprotein and that its phosphorylation state is regulated by an intracellular signaling pathway initiated by the transmembrane receptor Toll. Immunoblot analysis of cytoplasm from precisely staged embryos revealed that the phosphorylation state of dorsal is altered during the time period that Toll is activated. Moreover, mutations that constitutively activate Toll stimulated dorsal phosphorylation, while mutations that block Toll activation reduced the level of dorsal phosphorylation. We further demonstrated that signal-dependent dorsal phosphorylation is modulated by three intracellular proteins, pelle, tube, and cactus. Using double-mutant embryos, we then explored the nature of the kinase activity responsible for dorsal phosphorylation. We found that free dorsal is a substrate for a signal-independent kinase activity. In addition, our results imply that dorsal is a substrate for a Toll-dependent kinase. These results are consistent with the hypothesis that phosphorylation of Rel-related proteins may be required for the proper nuclear localization and transcriptional activity of these proteins.**

The dorsoventral axis of *Drosophila* embryos is defined by a nuclear concentration gradient of dorsal, a member of the Rel family of transcription factors (reviewed in references 23 and 42). Another member of the Rel family, NF-κB, mediates aspects of the immune and inflammatory responses in mammalian lymphocytes (reviewed in reference 3). The transcriptional activities of *Drosophila* dorsal and mammalian NF-κB are governed by a remarkably conserved mechanism. In both cases, extracellular signals promote the rapid redistribution of the Rel protein from the cytoplasm to the nucleus, where it then activates or represses target genes.

The posttranslational mechanism underlying the regulated nuclear translocation of Rel proteins has been most thoroughly characterized for NF-κB. Prior to its activation by extracellular signals, NF-κB is sequestered in the cytoplasm in a latent state (4). Cytoplasmic retention of NF-κB is mediated by an inhibitory protein, IκB, which binds to and masks the nuclear localization sequence of NF-κB (5, 7). In responsive cells, a variety of cytokines and mitogens, including interleukin-1 (IL-1) and phorbol esters, stimulate the nuclear import of NF-κB (33, 36, 45). These agents initiate signal transduction cascades that culminate in the dissociation of the NF-κB–IκB complex and the subsequent translocation of NF-κB into the nucleus (4).

Biochemical, genetic, and molecular biological evidence all suggests that the nuclear translocation of dorsal is controlled by a mechanism similar to that for NF-κB (54). In precellular *Drosophila* embryos, dorsal nuclear import is triggered by the ventrally restricted activation of a transmembrane receptor, Toll (14, 24, 25, 50). In response to Toll activation, dorsal protein rapidly migrates into nuclei on the ventral side of the embryo, while remaining excluded from the dorsal-most nuclei (40, 41, 52). A nuclear concentration gradient of dorsal protein that defines the fates of cells along the dorsoventral circumference is thereby established (53).

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Loss-of-function mutations in *Toll* disrupt intracellular signaling (1). As a result, dorsal remains in the cytoplasm throughout embryogenesis (40, 52). Although the nature of the signaling pathway initiated by Toll is unknown, at least three genes function intracellularly to regulate dorsal import. *tube* and *pelle* play positive roles in transmission of the ventral signal (2, 27). Loss of activity of *tube* or *pelle* has the same effect as the loss of *Toll*: nuclear translocation of dorsal is blocked (40). In contrast, loss-of-function mutations in a third gene, *cactus*, have an opposite effect (44). In *cactus* embryos, dorsal protein migrates into nuclei along the entire dorsoventral circumference, regardless of the activation state of Toll (40, 41, 52). These results suggest that cactus serves to secure dorsal in the cytoplasm and prevent its nuclear import in the absence of signaling, analogous to the function served by IκB in mammalian cells. Indeed, a physical interaction between dorsal and cactus has been demonstrated by immunoprecipitation experiments and by DNA-binding assays (17, 30).

Components of the NF-κB and dorsal activation pathways resemble each other molecularly as well as functionally. The dorsal protein is homologous to the p50 and p65 subunits of NF-κB (reviewed in reference 9). The cactus protein also shares a high degree of sequence similarity with its mammalian counterpart, IκB (17, 30). Finally, the cytoplasmic domain of Toll shares a modest degree of sequence similarity with the type I receptor for IL-1, a known physiological activator of NF-κB (16, 43). The sequence conservation between the receptors and final effectors of these two signaling pathways suggests that the intervening signal transduction components may be conserved as well.

The nature of the signaling pathways activated by the IL-1 receptor and by Toll remains unclear. Signal transduction motifs have not been identified in either the IL-1 receptor or Toll cytoplasmic domain. Nevertheless, both receptors appear to act through intracellular protein kinases. In mammalian cells, activation of the IL-1 receptor stimulates a variety of protein kinases, including cyclic AMP (cAMP)-dependent protein kinase and MAP-2 kinase, though the role of these

kinases in vivo is the subject of lively debate (8, 11, 48). Biochemical evidence suggests that both NF-κB and IκB may be targets for intracellular phosphorylation. Tumor necrosis factor alpha and IL-1 both stimulate the phosphorylation of IκB in vivo (6), while phosphorylation of purified IκB in vitro has been shown to block its ability to inhibit NF-κB (18). In addition, pharmacological agents that activate cAMP-dependent protein kinase or protein kinase C can induce NF-κB DNA-binding activity (10, 18, 47). In *Drosophila* embryos, a role for phosphorylation in the dorsoventral pathway has been clearly established: the *pelle* gene, which encodes a protein kinase (46), is required downstream of Toll for dorsal nuclear import. Furthermore, in cultured *Drosophila* cells, the coexpression of dorsal with the cAMP-dependent protein kinase catalytic subunit leads to an increase in dorsal's level of nuclear uptake and its transcriptional activity (35). Thus, like NF-κB and IκB, dorsal and cactus are prime candidates for regulation by phosphorylation.

In this report, we demonstrate that dorsal is a phosphoprotein and that the phosphorylation state of dorsal is dynamic in early embryos. Using a combined genetic and biochemical approach, we show that the activation of Toll stimulates an increase in the extent of dorsal phosphorylation. We explore this result further by characterizing the dependence of dorsal phosphorylation on the activities of tube, pelle, and cactus.

## MATERIALS AND METHODS

**Stocks.** Oregon R was used as the wild-type stock. The *tube* null allele, $tub^{R5.6}$ (27), was generously provided to our laboratory by Kathryn Anderson. *Df(3R)XM3* and *Df(3R)IR16* have been described previously (31, 46). All other mutations and balancers have been described by Lindsley and Zimm (32).

**Production of dorsal antiserum.** Full-length dorsal protein was expressed in *Escherichia coli* JM109(DE3) by isopropyl-β-D-thiogalactopyranoside (IPTG) induction of plasmid pARdl (41). For preparation of antigen, inclusion bodies were purified as described by Rio et al. (38). dorsal was further purified by electrophoretic separation on 3-mm-thick sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. Acrylamide strips containing dorsal protein were excised from the gel after staining with 4 M sodium acetate (28). For injection, an acrylamide strip containing approximately 500 μg of dorsal protein was homogenized in a small volume of phosphate-buffered saline (7 mM $Na_2HPO_4$, 3 mM $NaH_2PO_4$, 30 mM NaCl [pH 7.4]), using a Dounce homogenizer with a motor-driven pestle. The homogenized band was mixed with an equal volume of Freund's complete adjuvant (Gibco) and emulsified by sonication with a fine-tip probe. Rabbits were initially injected with 500 μg of dorsal protein and were bled 10 days after injection. Thereafter, rabbits were injected on a 2- to 4-week schedule with 250 μg of dorsal protein in acrylamide that was homogenized and emulsified with Freund's incomplete adjuvant (Gibco). All bleedings were performed 10 to 14 days postinjection.

**Preparation of crude embryonic lysates and molecular weight conversion by endogenous enzymes.** Embryos were collected, dechorionated, and stored at −70°C prior to use. Dechorionated frozen embryos (500 μl) were lysed in 1.5 ml of ice-cold lysis buffer (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% Triton X-100, 0.2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 1 μM pepstatin, 0.3 μM aprotinin [pH 7.5]) in a Dounce homogenizer, using 30 strokes of a tight-fitting pestle. Unlysed embryos and some yolk material were removed by filtration through 150-μm-pore-size Nytex mesh. A portion of the lysate was immediately mixed with an equal volume of 2× sample buffer (125 mM Tris, 20% glycerol, 4% SDS, 10% β-mercaptoethanol [pH 6.8]) and heated to 100°C for 5 min to stop endogenous enzymatic activity. Molecular weight conversion of dorsal by endogenous enzymes was carried out by incubating the remaining lysate at 30°C for 60 min, in the presence or absence of phosphatase inhibitors. On completion of the incubation, lysates were mixed with an equal volume of 2× sample buffer and heated to 100°C for 5 min. Prior to electrophoresis, all samples were centrifuged at 10,000 × *g* for 5 min at 4°C to remove insoluble material.

**Immunoblot analysis.** Proteins were fractionated on SDS–11.5% polyacrylamide gels containing a 120:1 ratio of acrylamide to bisacrylamide. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) in 15.6 mM Tris–120 mM glycine. Electrotransfer was carried out for 2 h at 100 V (25 V/cm), using ice-cold buffer. We used the Bio-Rad Mini-Protean system for electrophoresis and transfer. Immunoblotting was performed by the chemiluminescence method of Gillespie and Hudspeth (20), with slight modifications. Membranes were blocked for 1 h in 6% casein–1% polyvinylpyrrolidone 40–59 mM $Na_2HPO_4$–16 mM $NaH_2PO_4$–68 mM NaCl–0.02% $NaN_3$ (pH 7.2). Following blocking, blots were incubated with rabbit polyclonal antidorsal serum for 1 h. Before use, the crude serum was diluted in 59 mM $Na_2HPO_4$–16 mM $NaH_2PO_4$–0.5 M NaCl–0.5% Tween 20 (pH 7.4) and preadsorbed to protein derived from 0- to 3-h *dorsal* null embryos [maternal genotype $dl^1/dl^1$ or $In(2L)dl^{H1}/Df(2L)TW119$] that had been coupled to cyanogen bromide-activated Sepharose. This preadsorbed serum was used at a dilution of 1/10,000 (relative to the original serum) in blocking buffer. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Calbiochem) was used at a dilution of 1/20,000 in blocking buffer. Alkaline phosphatase activity was detected by using 0.4 mM 3-(4-methoxyspiro-{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1$^{3,7}$]decan}-4-yl)-phenylphosphate (AMPPD) (Tropix) or 0.25 mM 3-(4-methoxyspiro{1,2-dioxetane-3,2′-tricyclo-[3.3.1.1$^{3,7}$]decan}-4-yl)phenyl phosphate (CSPD) (Tropix) in 50 mM 2-amino-2-methyl-1-propanol–1 mM $MgCl_2$–10% Sapphire enhancer (Tropix) (pH 10). All incubations were performed at room temperature.

**Preparation of biotinylated embryonic protein.** Dechorionated frozen embryos (500 μl) were homogenized in 1.5 volumes of ice-cold lysis buffer in a Dounce homogenizer. Unlysed material was removed by filtration through 150-μm-pore-size Nytex mesh. The crude lysates were cleared by centrifugation at 100,000 × *g* for 1 h at 4°C. The lipid-depleted, clear middle layer was removed with a finely drawn out Pasteur pipette and stored at −70°C. Biotinylation was carried out as described by Gillespie and Hudspeth (19, 20), with several modifications. Protein extracts were diluted to 1 mg/ml in 25 mM HEPES (pH 8.0). If necessary, the pH of the diluted protein extract was adjusted to 8.0. Immediately before use, a 5 mM solution of *N*-hydroxysulfosuccinimidobiotin (sulfo-NHS-biotin) (Pierce) was prepared in 25 mM HEPES (pH 8.0). To initiate labeling, an equal volume of 5 mM sulfo-NHS-biotin was mixed with the 1-mg/ml embryonic extract. Reactions were carried out at room temperature for 15 to 30 min. The biotinylation reaction was stopped by the addition of 1 M lysine (pH 9) to a final concentration of 20 mM. Biotinylated proteins were stored at −70°C, at which temperature they appeared to be stable for at least 6 months. The labeling reaction did not appreciably alter the molecular weight of dorsal, as determined by immunoblotting.

**Immunoprecipitation and alkaline phosphatase assays.** Biotinylated extracts (10 to 20 μg of protein) were diluted into 500 μl of lysis buffer containing, additionally, 10 mg of bovine serum albumin per ml and 0.08% SDS. All incubations were performed at 4°C. Extracts were first precleared with 25 μl (wet volume) of protein A-Sepharose (Sigma) for 1 h and then incubated overnight with 3 μl of crude rabbit preimmune serum. Preimmune antibody-antigen complexes were removed by incubation with 25 μl of protein A-Sepharose. Extracts cleared with preimmune serum were then incubated with 3 to 10 μl of polyclonal rabbit antidorsal serum. This serum had been first preadsorbed to protein derived from *dorsal* null embryos as described for immunoblotting except that after preadsorption, the serum was concentrated to its original volume in Centricon C-30 concentrators (Amicon). Incubation with postimmune serum was carried out for 2 h. Postimmune antibody-antigen complexes were recovered by incubation with 25 to 50 μl of protein A-Sepharose for 1 h. Preimmune and postimmune antibody-antigen complexes were washed twice with 50 mM Tris–0.5% Triton X-100–300 mM NaCl (pH 7.5), twice with the same solution containing 500 mM NaCl, and twice with 50 mM Tris–100 mM NaCl–1 mM $MgCl_2$–1 mM $CaCl_2$–1% Triton X-100–1% sodium deoxycholate–0.25% SDS (pH 7.5). Complexes were then washed with 10 mM Tris (pH 7.5). Immunoprecipitated proteins were eluted with 40 μl of 1× sample buffer and heated to 100°C for 5 min.

Dephosphorylation of immunoprecipitated proteins by alkaline phosphatase was carried out prior to elution of proteins from the antibody-protein A-Sepharose beads. After washing in 10 mM Tris (pH 7.5), pellets were washed twice with CIP buffer (10 mM Tris, 10 mM $MgCl_2$, 100 mM KCl [pH 8]) and resuspended in 50 μl of CIP buffer. Thirty units of calf alkaline phosphatase (Boehringer Mannheim) was added to appropriate tubes with or without 50 mM β-glycerophosphate and 2 mM sodium vanadate. Phosphatase reactions were carried out for 1 h at 37°C. Following the incubation, pellets were washed in 10 mM Tris (pH 7.5), and proteins were eluted as indicated above.

Immunoprecipitated proteins were separated on SDS-polyacrylamide gels and transferred to charged nylon membranes (Zeta-Probe; Bio-Rad) as described above for immunoblotting. Biotinylated proteins were detected as described by Gillespie and Hudspeth (20), using a 1/40,000 dilution of streptavidin-alkaline phosphatase (TAGO) and the chemiluminescent substrate AMPPD.

**Micropipette collection of total embryonic cellular contents.** Embryos were collected, dechorionated, and lined up on double-sided cellophane tape. Embryos were overlaid with halocarbon oil and staged by eye according to the conventions of Campos-Ortega and Hartenstein (13). Stage 2 embryos were visually identified by anterior and posterior embryonic retraction from the vitelline membrane. Stage 5 embryos were identified as those undergoing cellularization. Only those embryos in which membrane invagination was between 40 and 60% complete were used for this study. The entire contents of five to seven embryos were extracted with a microinjection needle, leaving behind only the deflated husk of vitelline membrane. This extract was then transferred into 20 μl of 1× sample buffer containing, additionally, 1 mM EDTA, 50 mM NaF, 5 μM microcystin-LR, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 1 μM pepstatin, and 0.3 μM aprotinin and was kept on ice. The contents of additional pools of 5 to 7 embryos were collected and transferred into the same 20 μl of buffer until the contents of 20 embryos had been collected. The average time that extracted material resided in a microinjection needle was 2.5 min. Once the cellular contents of 20 embryos had been collected, samples were immediately heated to 100°C for 5 min.

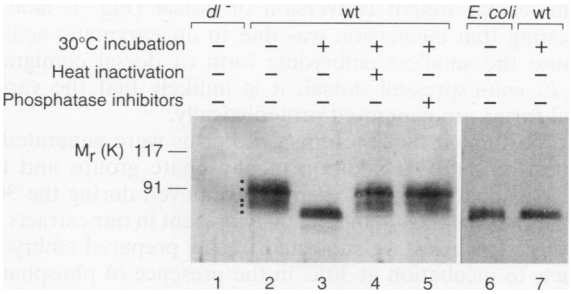


FIG. 1. Posttranslational modification of dorsal in early embryos. Crude lysates were prepared from 0- to 3-h *dorsal* null [*In(2L)dl*$^{H}$/*Df(2L)TW119*] embryos (*dl*$^{-}$; lane 1), from 0- to 3-h wild-type embryos (wt; lanes 2 to 5 and 7), or from *E. coli* expressing dorsal from plasmid pARdl (lane 6). Embryonic protein (40 μg) or *E. coli* protein (1 μg) was fractionated on an SDS–11.5% polyacrylamide gel containing a 120:1 ratio of acrylamide to bisacrylamide. Proteins were electrophoretically transferred to membranes and detected with polyclonal antidorsal serum. In lanes 1 and 2, lysates were immediately mixed with an equal volume of 2× sample buffer and incubated for 5 min at 100°C to stop enzyme activity. Small dots indicate five dorsal isoforms. Occasionally, the second-largest band can be resolved as a doublet. In lane 3, lysate was incubated for 1 h at 30°C to allow molecular weight conversion of dorsal. In lane 4, lysate was heated to 100°C for 3 min to denature proteins prior to incubation for 1 h at 30°C. In lane 5, phosphatase inhibitors (80 mM β-glycerophosphate and 20 mM sodium $PP_i$) were added to the lysate prior to incubation for 1 h at 30°C. Following incubation at 30°C, crude lysates in lanes 3 to 5 were mixed with an equal volume of 2× sample buffer and heated to 100°C for 5 min. Lane 6, full-length *E. coli*-expressed dorsal protein; lane 7, same embryonic lysate as lane 3. Prestained protein molecular weight markers (Bio-Rad high-molecular-weight standards) were calibrated against unstained marker proteins: $M_r$ 117,000, prestained β-galactosidase; $M_r$ 91,000, prestained serum albumin.

## RESULTS

**Multiple isoforms of dorsal are present in wild-type embryonic extracts.** The dorsal protein appears as a broad doublet on immunoblots of embryonic extracts separated on standard SDS-polyacrylamide gels (21, 40, 52). To refine the electrophoretic separation of dorsal, we fractionated crude embryonic lysates on SDS-polyacrylamide gels containing low bisacrylamide concentrations. Upon immunoblotting with antidorsal serum, these higher-resolution gels revealed additional heterogeneity in dorsal's apparent molecular weight. The dorsal protein comprises an ensemble of at least five immunoreactive bands in 0- to 3-h wild-type embryos (i.e., embryos 0 to 3 h in age) (Fig. 1, lane 2; see also Fig. 4, lane 4). The fastest-migrating, very faint band has a relative molecular weight of 76,000, similar to the predicted molecular weight of 75,475 (51, 52). The four more slowly migrating bands have apparent molecular weights ranging from 79,000 to 97,000.

In the course of characterizing dorsal, we found that incubation of crude embryonic extracts at 30°C altered the profile of the dorsal ensemble. Such an incubation converted the dorsal isoforms to a single, faster-migrating band with an intensity greater than that of any of the original bands (Fig. 1, lane 3). This band comigrated with the smallest dorsal isoform in extracts from 0- to 3-h embryos and with full-length dorsal expressed in *E. coli* (Fig. 1, lanes 2, 3, 6, and 7). Heat inactivation of extracts prior to incubation at 30°C inhibited

the molecular weight conversion of dorsal (Fig. 1, lane 4), suggesting that conversion was due to an enzymatic activity. Because the smallest embryonic form of dorsal comigrated with *E. coli*-expressed dorsal, it is unlikely that the variant dorsal forms are generated proteolytically.

We postulated that the dorsal isoforms were generated by the posttranslational addition of phosphate groups and that these phosphate groups were being removed during the 30°C incubation by a phosphatase activity present in our extracts. To test this hypothesis, we subjected freshly prepared embryonic extracts to incubation at 30°C in the presence of phosphatase inhibitors. A mixture of two broad-specificity phosphatase inhibitors, β-glycerophosphate and sodium $PP_i$, completely suppressed the conversion of the dorsal bands by the endogenous activity (Fig. 1, lane 5). The ability of phosphatase inhibitors to block the removal of posttranslational modifications on dorsal supports our hypothesis that dorsal is a phosphoprotein and that the endogenous converting activity is a phosphatase.

**dorsal is modified by phosphorylation.** Although broad-specificity phosphatase inhibitors prevent the conversion of dorsal species to faster-migrating forms, the activity of the responsible enzyme has not been defined in vitro. To demonstrate conclusively that dorsal is modified by phosphorylation, we assayed the effect of purified alkaline phosphatase on immunoprecipitated dorsal protein. For these experiments, we labeled embryonic protein extracts with sulfo-NHS-biotin. We chose this nonradioactive technique for its ability to label embryonic proteins regardless of whether they are synthesized in the embryo or in the ovary. The choice of this labeling technique also allowed us to take advantage of a sensitive detection protocol for biotinylated proteins (20).

As shown in Fig. 2A, we recovered two proteins from extracts of wild-type 0- to 3-h embryos by immunoprecipitating with antidorsal serum (Fig. 2A, lane 4). Neither protein was immunoprecipitated with preimmune serum. The larger protein migrated with an apparent molecular weight of 94,000, within the range expected for dorsal. The 94,000-$M_r$ protein was immunoprecipitated from extracts denatured by heating in 1% SDS (Fig. 2A, lane 6) but not from extracts prepared from *dorsal* null embryos (Fig. 2A, lane 2), confirming its identification as dorsal. Incubation of immunoprecipitated dorsal protein at 37°C for 1 h did not alter its mobility (Fig. 2B, lane 1). Incubation of the precipitated protein with purified alkaline phosphatase, however, converted the dorsal ensemble to a faster-migrating species (Fig. 2B, lanes 2 and 4). Treatment of immunoprecipitated protein with potato acid phosphatase generated similar results (data not shown). The molecular weight conversion of dorsal by alkaline phosphatase was blocked by 50 mM β-glycerophosphate and 2 mM sodium vanadate (Fig. 2B, lanes 3 and 5). The added phosphatase was therefore responsible for the observed conversion. From these results, we conclude that dorsal is modified by phosphorylation in precellular embryos.

The protein that coprecipitates with dorsal (Fig. 2A, lane 4) has an approximate molecular weight of 72,000 (p72). p72 was not immunoprecipitated with antidorsal serum from denatured wild-type extracts (Fig. 2A, lane 6) or from *dorsal* null extracts (Fig. 2A, lane 2), suggesting that p72 is a noncovalently associated protein and not a degradation product of dorsal. Like that of dorsal, the mobility of p72 was increased by phosphatase treatment of the immunoprecipitated complex (Fig. 2B, lanes 2 and 4) and the mobility shift was blocked by the addition of phosphatase inhibitors (Fig. 2B, lanes 3 and 5). Kidd (30) has demonstrated the association of dorsal and an embryonic phosphoprotein with an $M_r$ of 69,000 to 75,000,

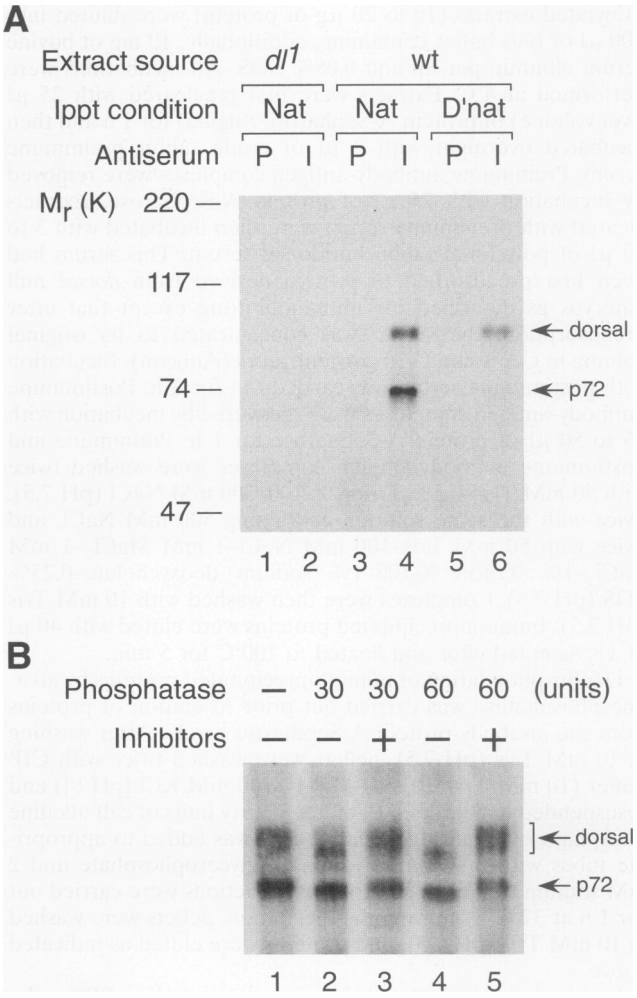


FIG. 2. Molecular weight conversion of immunoprecipitated dorsal and p72 by alkaline phosphatase. (A) Specificity of the immunoprecipitation (Ippt) reaction. Embryonic protein labeled with sulfo-NHS-biotin was immunoprecipitated with rabbit preimmune serum (lanes 1, 3, and 5) or rabbit polyclonal antidorsal serum (lanes 2, 4, and 6). Proteins were immunoprecipitated from extracts of *dorsal* null 0- to 3-h embryos (maternal genotype $dl^1/dl^1$) (lanes 1 and 2), wild-type (wt) 0- to 3-h embryos (lanes 3 and 4), or wild-type 0- to 3-h embryos denatured by boiling in 1% SDS (lanes 5 and 6). Immunoprecipitated proteins were separated on standard SDS–10% polyacrylamide gels, transferred to charged nylon membranes, and detected with streptavidin-alkaline phosphatase. P, preimmune serum; I, immune serum; Nat, native extracts; D'nat, denatured extracts. Prestained protein molecular weight markers (Bio-Rad low-molecular-weight standards) were calibrated against unstained marker proteins: $M_r$ 220,000, prestained myosin; $M_r$ 117,000, prestained β-galactosidase; $M_r$ 74,000, prestained serum albumin; $M_r$ 47,000, prestained ovalbumin. (B) Alkaline phosphatase conversion of dorsal and p72. Biotinylated embryonic extracts were subjected to immunoprecipitation with antidorsal serum. Immunoprecipitated proteins were incubated at 37°C for 1 h in the absence of enzyme (lane 1), in the presence of alkaline phosphatase (lanes 2 and 4), or in the presence of alkaline phosphatase plus phosphatase inhibitors (50 mM β-glycerophosphate and 2 mM sodium vanadate) (lanes 3 and 5). Proteins in lanes 2 and 3 were incubated with 30 U of alkaline phosphatase; proteins in lanes 4 and 5 were incubated with 60 U of alkaline phosphatase. Proteins were fractionated on 12% polyacrylamide gels containing a 120:1 ratio of acrylamide to bisacrylamide and were detected as in panel A. For reasons discussed in Results, we believe that p72 is cactus.

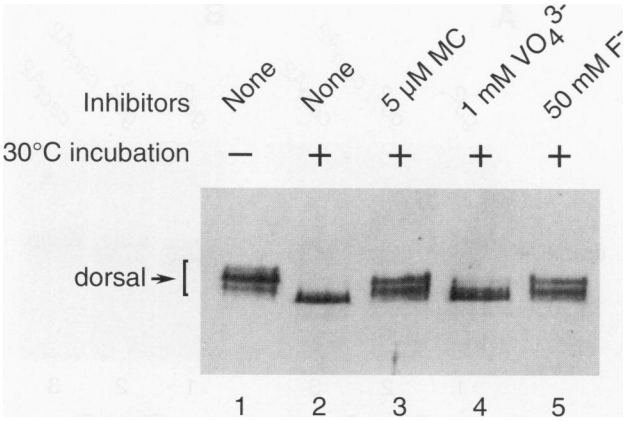


FIG. 3. The molecular weight conversion of dorsal is blocked by inhibitors specific for serine/threonine phosphatases. Crude lysates were prepared from wild-type 0- to 3-h embryos. Embryonic protein (40 μg) was analyzed by immunoblotting with antidorsal serum as in Fig. 1. In lane 1, lysate was immediately mixed with an equal volume of 2× sample buffer and incubated for 5 min at 100°C to stop enzyme activity. In lane 2, lysate was incubated for 1 h at 30°C. In lanes 3 to 6, phosphatase inhibitors were added to lysates prior to incubation. Lane 3, 5 μM microcystin-LR; lane 4, 1 mM sodium vanadate; lane 5, 50 mM sodium fluoride.

identified as the product of the *cactus* gene. Therefore, we believe that p72 is cactus.

**dorsal is phosphorylated on serine or threonine residues.** To further characterize dorsal phosphorylation, we exploited the phosphatase activity present in our crude embryonic lysates. A variety of phosphatase inhibitors were assayed for the ability to block the molecular weight conversion of dorsal in embryonic lysates. Fluoride ion ($F^-$) preferentially inhibits serine/threonine phosphatases, while orthovanadate ($VO_4^{3-}$) is selective for tyrosine phosphatases (12, 22). We found that sodium fluoride (50 mM) strongly inhibited dorsal's molecular weight conversion by the embryonic phosphatase (Fig. 3, lane 5). In contrast, sodium vanadate (1 mM) had little effect on the conversion of dorsal (Fig. 3, lane 4). These results suggested that the primary dorsal-converting activity in our extracts was a serine/threonine phosphatase. We confirmed this hypothesis by using in the conversion assay microcystin-LR, a specific inhibitor of serine/threonine phosphatases (34). The molecular weight conversion of dorsal was almost completely inhibited by 5 μM microcystin-LR (Fig. 3, lane 3). We conclude from these experiments that most or all of the posttranslational modifications on dorsal are serine- or threonine-linked phosphates, although it is possible that some tyrosine residues may also be modified.

**Transduction of the ventral signal alters the state of dorsal phosphorylation.** Having shown that dorsal is phosphorylated in 0- to 3-h embryos, we examined whether the phosphorylation state of dorsal changes during this time period. We were particularly interested in determining whether the phosphorylation state of dorsal was regulated by the ventral signal transmitted from Toll. Toward this end, we established two criteria by which to judge whether any changes that we might observe in dorsal phosphorylation were dependent on Toll activation.

First, the phosphorylation state of dorsal should be altered in embryos derived from mothers carrying mutations that disrupt the signal transduction process. A recessive dorsalizing

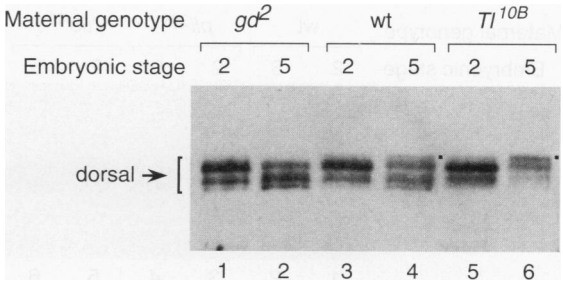


FIG. 4. Signal-dependent phosphorylation of dorsal protein. The total cellular contents of individual embryos were collected by microinjection needle. Pools of staged extracts from 20 embryos were analyzed by immunoblotting with antidorsal serum. Stage 2 embryos (lanes 1, 3, and 5) were undergoing early cleavage divisions. Stage 5 embryos (lanes 2, 4, and 6) were in the process of cellularization. Extracts were collected from embryos with maternal genotypes $gd^2$ (lanes 1 and 2), wild type (wt) (lanes 3 and 4), and $Tl^{10B}$/+ (lanes 5 and 6). A small dot marks the position of the most slowly migrating form of dorsal in wild-type and $Tl^{10B}$ embryos (lanes 4 and 6, respectively). This band is absent in $gd^2$ embryos (lane 2).

allele of the *gastrulation defective* gene, $gd^2$, blocks production of the extracellular Toll ligand (26, 49). Consequently, signal transduction is not initiated and dorsal remains cytoplasmic in these embryos. In contrast, a dominant ventralizing allele of the Toll receptor, $Tl^{10B}$, produces a constitutively active receptor (43). As a result, dorsal undergoes nuclear translocation along the entire dorsoventral circumference. A comparison of the dorsal ensemble in wild-type, $gd^2$, and $Tl^{10B}$ embryos (that is, embryos derived from wild-type, $gd^2/gd^2$, and $Tl^{10B}$/+ females) should allow us to discriminate between signal-dependent and signal-independent changes in the phosphorylation state of dorsal.

Changes in dorsal phosphorylation should also satisfy a second criterion if they are to be considered signal dependent: the phosphorylation state change should occur in the narrow developmental time period during which the ventral signal is transmitted. Immunocytochemical analysis indicates that the graded movement of dorsal into nuclei begins in embryonic stage 3, about 80 min after fertilization, and is complete by stage 5, 130 min after fertilization (40, 41, 52). Microinjection experiments demonstrate that the subcellular localization of dorsal can be altered by an exogenous ligand applied during embryonic stages 3 and 4 (50). As defined by these two classes of experiments, signal transduction occurs predominantly during embryonic stages 3 and 4. We therefore compared the dorsal ensemble in stage 2 embryos (the presignaling state) to that present in stage 5 embryos (the postsignaling state). The standard method for collecting timed embryos in bulk (56) did not yield embryos that were staged with enough precision for these experiments; such collections are imperfectly synchronized and are contaminated with variable numbers of unfertilized eggs. To prepare precisely staged embryonic extracts, we therefore collected embryos and visually determined their age according to the morphological landmarks described by Campos-Ortega and Hartenstein (13). Using a microinjection needle, we then extracted the entire cellular contents of individual embryos. Total cellular extracts from embryos at identical developmental stages were pooled and analyzed by immunoblotting with antidorsal serum (Fig. 4).

In extracts from wild-type stage 2 embryos, multiple dorsal species are already present (Fig. 4, lane 3). The signaling pathway that regulates dorsal nuclear import is not active in

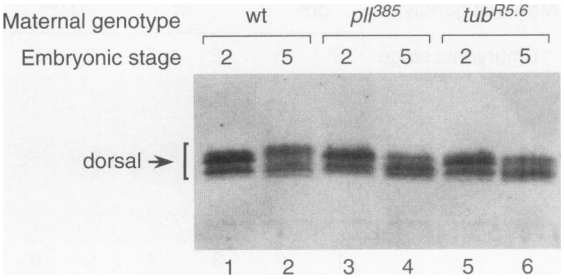


FIG. 5. Loss-of-function mutations in *tube* or *pelle* block the signal-dependent phosphorylation of dorsal. Immunoblot analysis of total cellular contents extracted from stage 2 embryos (lanes 1, 3, and 5) and stage 5 embryos (lanes 2, 4, and 6) was carried out as for Fig. 4. Extracts were collected from wild-type (wt) embryos (lanes 1 and 2) or embryos with maternal genotype $pll^{385}$/*Df(3R)IR16* (lanes 3 and 4) or $tub^{R5.6}$/*Df(3R)XM3* (lanes 5 and 6).

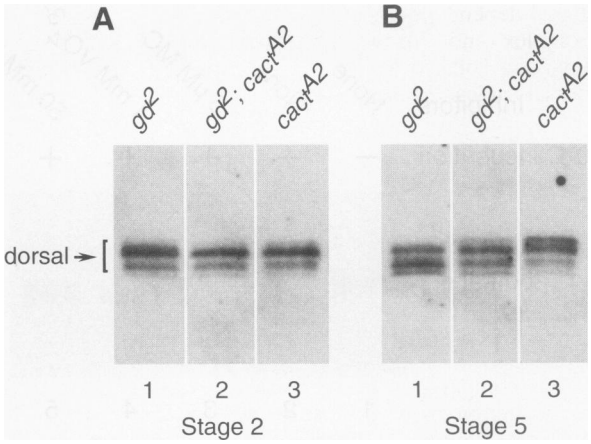


FIG. 6. Inactivation of cactus stimulates dorsal phosphorylation. Immunoblot analysis of total cellular contents extracted from stage 2 (A) and stage 5 (B) embryos was carried out as for Fig. 4. Extracts were collected from embryos with maternal genotype $gd^2$ (lane 1), $gd^2$;$cact^{A2}$ (lane 2), or $cact^{A2}$ (lane 3).

these early embryos. Therefore, dorsal phosphorylation at stage 2 appears to be independent of signaling. This result is confirmed by our analysis of stage 2 extracts obtained from dorsalized ($gd^2$) and ventralized ($Tl^{10B}$) embryos. At this stage, the profiles of immunoreactive bands in $gd^2$ and $Tl^{10B}$ embryos are essentially identical to that of wild-type embryos (compare Fig. 4, lanes 1 and 5, with Fig. 4, lane 3, and Fig. 5, lane 1).

Following the activation of intracellular signaling by Toll (stage 5), the dorsal ensemble is altered in wild-type, $Tl^{10B}$, and $gd^2$ embryos. In wild-type embryos, the dorsal ensemble broadens at stage 5. Faster-migrating and more slowly migrating forms of dorsal appear that were not visible in stage 2 extracts (Fig. 4; compare lanes 4 and 3). In addition, the intensities of the intermediate bands decrease in conjunction with the appearance of the new dorsal isoforms. In striking contrast to wild-type embryos, ventralized $Tl^{10B}$ embryos contain a preponderance of more slowly migrating forms of dorsal at stage 5 but lack faster-migrating forms (Fig. 4, lane 6). The largest isoform of dorsal in stage 5 $Tl^{10B}$ embryos comigrates with that in stage 5 wild-type embryos. Like the dorsal isoforms in 0- to 3-h wild-type extracts, the dorsal isoforms in stage 5 $Tl^{10B}$ embryos can be converted to a faster-migrating form by incubation at 30°C, and this conversion is blocked by microcystin-LR (data not shown). Whereas the most slowly migrating isoform of dorsal is enhanced in $Tl^{10B}$ embryos, we found that this isoform is missing in dorsalized $gd^2$ embryos (Fig. 4, lane 2) and in dorsalized $pipe^1$ embryos (data not shown). In these ligand-deficient embryos, faster-migrating forms of dorsal become more prominent at stage 5.

It is possible that faster-migrating and more slowly migrating dorsal bands reflect a rearrangement of phosphate groups on the dorsal protein and that no net shift in the stoichiometry of phosphorylation has occurred. However, the simplest interpretation of the mobility shifts that we observed is that faster-migrating isoforms arise from dephosphorylation while more slowly migrating isoforms arise from phosphorylation. Thus, our results suggest that dorsal is phosphorylated in response to Toll activation and that dephosphorylation occurs in the absence of Toll activation. In wild-type embryos, in which the Toll-activated and Toll-inactivated states are represented (ventral and dorsal sides of the embryo, respectively), dorsal protein appears to undergo both phosphorylation and dephosphorylation.

**Signal-dependent dorsal phosphorylation is blocked by mutations in *pelle* and *tube*.** The products of two genes, *pelle* and *tube*, are required intracellularly for dorsal nuclear translocation (27, 31, 39, 40, 46). We examined the role of these genes in the regulation of dorsal phosphorylation. dorsal's phosphorylation state was analyzed by immunoblotting the total cellular contents collected from embryos hemizygous for a strong mutation in *pelle* or for a null mutation in *tube*. The dorsal protein in *pelle* or *tube* stage 2 embryos resembles that in wild-type stage 2 embryos (Fig. 5, lanes 1, 3, and 5). At stage 5, however, the most slowly migrating form of dorsal seen in wild-type embryos is absent in *pelle* and *tube* embryos (Fig. 5, lanes 2, 4, and 6). Furthermore, dorsal phosphorylation in these mutant backgrounds was less at stage 5 than in wild-type embryos. The dorsal phosphorylation states in *pelle* and in *tube* mutant embryos closely resembled that of dorsal in *gastrulation defective* embryos (Fig. 5, lanes 3 to 6; compare with Fig. 4, lanes 1 and 2). We conclude that *pelle* and *tube* are both required for the signal-dependent phosphorylation of dorsal.

**Loss of *cactus* function stimulates dorsal phosphorylation.** Loss-of-function mutations in the *cactus* gene, in contrast to those in *pelle* or *tube*, greatly expand the domain of dorsal nuclear translocation. We examined dorsal protein in embryos produced by females carrying the strongest viable *cactus* allele, $cact^{A2}$. Although it is not null (39), this mutation appears to greatly diminish the association of dorsal and cactus (30). The stage 2 dorsal ensemble in these embryos resembles the stage 2 dorsal ensemble seen in all other wild-type and mutant embryos (Fig. 6A, lane 3). At stage 5, however, the proportion of more slowly migrating dorsal isoforms is unambiguously increased relative to the wild-type level, similar to results obtained for $Tl^{10B}$ (Fig. 6B, lane 3; compare with Fig. 4, lane 4, and Fig. 5, lane 2). Thus, dorsal phosphorylation increases under conditions in which the affinity of cactus for dorsal is reduced. These results raise the possibility that free dorsal is a substrate for phosphorylation.

We next explored the nature of the kinase responsible for the signal-dependent phosphorylation of dorsal. The phosphorylation of dorsal could simply be catalyzed by a Toll-stimulated kinase acting on either free or cactus-bound dorsal. In this case, dorsal phosphorylation should be strictly dependent on Toll's activation state. Alternatively, signal-dependent phosphorylation could arise by a signal-independent kinase acting on free dorsal: such a kinase activity would seem to be

signal-dependent because the dissociation of the dorsal-cactus complex (and thus the appearance of free dorsal) is normally linked to Toll activation. In this second case, dissociation of the dorsal-cactus complex should induce phosphorylation, regardless of the activation state of Toll. To distinguish between these two possibilities, we examined dorsal in embryos derived from $gd^2;cact^{A2}$ double-mutant females. Like $gd^2$ females, $gd^2;cact^{A2}$ females produce embryos in which Toll activation is blocked. The added presence of the $cact^{A2}$ mutation, however, increases the concentration of free dorsal in the double-mutant embryos, even in the absence of signaling. Thus, dissociation of the dorsal-cactus complex is uncoupled from receptor activation in $gd^2;cact^{A2}$ mutants; such embryos display a lateralized phenotype (39).

We compared stage 2 extracts from $gd^2;cact^{A2}$ double-mutant embryos with those of $gd^2$ and $cact^{A2}$ single-mutant embryos. We saw no difference in the dorsal ensemble at this early stage, as expected (Fig. 6A). However, when we examined stage 5 extracts from the $gd^2;cact^{A2}$ embryos, we were clearly able to distinguish the presence of a slowly migrating form of dorsal that was not present in $gd^2;cact^{A2}$ embryos at stage 2 or in $gd^2$ embryos at stage 5 (Fig. 6B, lanes 1 and 2; Fig. 6A, lane 2). This protein comigrates with the largest form of dorsal seen in the $cact^{A2}$ embryos, though its intensity is greatly reduced. These data indicate that the mutational inactivation of cactus is sufficient to induce some dorsal phosphorylation.

## DISCUSSION

**dorsal is an embryonic phosphoprotein.** Proteins of the Rel family are retained in the cytoplasm through their association with inhibitory proteins (reviewed in reference 3). In response to specific extracellular signals, the Rel-related proteins are released from their cytoplasmic inhibitors and translocate into the nucleus, where they act as transcriptional regulators. The biochemical trigger for dissociation of the cytoplasmic inhibitory proteins has not been identified in vivo but is thought to involve phosphorylation of the Rel protein or its inhibitor.

We have examined a *Drosophila* Rel homolog, dorsal, for evidence of posttranslational modification that might modulate its association with its cytoplasmic inhibitor, cactus. Using high-resolution immunoblots, we have demonstrated that multiple dorsal species are present in precellular embryos. The dorsal isoforms can be converted by embryonic phosphatases to a single band that comigrates with *E. coli*-expressed dorsal. Furthermore, the conversion of the dorsal isoforms can be completely inhibited by phosphatase inhibitors. Each of the isoforms distinguishable on our immunoblots therefore represents an alternatively phosphorylated form of the protein. Most, if not all, of these phosphates reside on serine or threonine residues.

The dorsal protein contains many potential sites for phosphorylation by serine/threonine kinases, including sites for cAMP-dependent kinase and protein kinase C (37). The majority of these sites are contained within amino acids 47 to 351, the Rel homology domain (51, 52), which includes sequences shown to be important for nuclear import, dimerization, and DNA binding of dorsal and other Rel family proteins (9, 29, 35). The Rel homology domain also encompasses the sequences required for cactus binding, amino acids 168 to 350 (30). Phosphorylation of serine or threonine residues within the Rel homology domain might therefore modulate a variety of dorsal's functions during embryogenesis, including its binding to cactus, its nuclear localization, or its transcriptional activity.

**The phosphorylation state of dorsal is altered in response to the axis-determining ventral signal.** The phosphorylation state of dorsal is not static but undergoes complex changes between embryonic stages 2 and 5, the same period during which dorsal translocates into nuclei in response to the ventral signal. At stage 2, several phosphorylated isoforms of dorsal are found in embryos, regardless of the embryo's dorsoventral phenotype. The phosphate groups present on dorsal in these early embryos may be added during oogenesis, since phosphorylated forms of dorsal are found in ovarian extracts (30). By stage 5, the nuclear dorsal gradient is complete in wild-type embryos. At this time, the dorsal ensemble is broadened by the appearance of new isoforms or newly prominent isoforms, both those that migrate more slowly and those that migrate faster than the dorsal isoforms at stage 2.

If the temporal changes in dorsal phosphorylation were induced by the ventral signal, we expected that dorsal phosphorylation would reflect the activation state of Toll. This expectation was affirmed: mutations that alter Toll's activity likewise alter the phosphorylation state of dorsal. In embryos with constitutively activated Toll receptors ($Tl^{10B}$), the more slowly migrating isoforms of dorsal dominate the stage 5 ensemble while faster-migrating isoforms of dorsal are absent. Contrasting with this result, ligand-deficient $gd^2$ embryos lack the most slowly migrating form of dorsal at stage 5. Instead, faster-migrating isoforms become more abundant. Although phosphorylation and dephosphorylation of dorsal both coincide with signal transduction, our results demonstrate that it is the phosphorylation of dorsal that is dependent on Toll activation.

The relative abundance of the most slowly migrating isoform of dorsal is less than the relative abundance of nuclear dorsal, as determined by immunocytochemistry (21, 40, 41, 52). Therefore, while the appearance of the most slowly migrating dorsal isoform correlates with dorsal's nuclear import, the nuclear form of dorsal is not represented solely by this isoform. No simple correlation appears to exist between the relative abundance of each of the dorsal isoforms and the subcellular distribution of the dorsal protein. The phosphorylation of a particular residue, rather than the overall stoichiometry of phosphorylation of dorsal, may be the critical stimulus for nuclear import; more than one band of the ensemble may be phosphorylated at this critical site.

The accumulation of dephosphorylated dorsal isoforms in stage 5 $gd^2$ embryos demonstrates that dorsal's phosphorylation state is also regulated by a mechanism that is independent of the ventral signal. As noted above, signal-independent dephosphorylation of dorsal occurs during the same time period as signal-dependent phosphorylation. Dephosphorylation could therefore play an important role in signal transduction; for example, dephosphorylation may be a prerequisite for dorsal to be phosphorylated at a signal-dependent site.

**tube and pelle are required for the signal-dependent phosphorylation of dorsal.** Genetic analyses have identified two genes, *tube* and *pelle*, that act downstream of Toll to positively regulate dorsal's nuclear import (27, 40). Dorsal phosphorylation is affected by mutations in both of these genes. Null mutations in *tube* or strong mutations in *pelle* block the signal-dependent phosphorylation of dorsal, implying that these genes promote the phosphorylation of dorsal in wild-type embryos. The tube protein does not resemble other proteins in the data base (31). Immunocytochemical data show that it is present in the cortical cytoplasm of the embryo and appears to be associated with or subjacent to the plasma membrane (21). This localization suggests that tube might interact with the cytoplasmic domain of Toll or with the cytoskeleton and

thereby mediate transmission of the ventral signal from Toll to other components in the signaling pathway. The *pelle* gene encodes a serine/threonine protein kinase (46). Genetic evidence suggests that pelle functions prior to dissociation of the dorsal-cactus complex (39). Because multiple dorsal species are seen in stage 2 *pelle* mutant embryos, the pelle protein kinase is not responsible for generating the dorsal isoforms present in these early embryos. However, pelle is required for transduction of the ventral signal (46). The pelle protein might stimulate dissociation of the dorsal-cactus complex directly, by phosphorylating either dorsal or cactus, or indirectly, by phosphorylating another component of the signaling pathway. An additional role for pelle following dissociation of the dorsal-cactus complex is also possible.

**dorsal is phosphorylated by signal-independent and signal-dependent kinase activities.** Mutations in *cactus*, as well as those in *tube* and *pelle*, affect the phosphorylation state of dorsal. We found that the proportion of more slowly migrating dorsal isoforms is substantially increased by the strong $cact^{A2}$ mutation. This mutation greatly decreases the affinity of cactus for dorsal and thereby necessarily increases the concentration of free dorsal in $cact^{A2}$ embryos (30). We therefore hypothesize that free dorsal is a substrate for phosphorylation and that the shift in dorsal phosphorylation reflects the increased quantity of free dorsal in $cact^{A2}$ embryos. Given this result, signal-dependent dorsal phosphorylation could be generated by two different types of kinase activity. In one case, dorsal might be a substrate for a Toll-activated kinase, either before or after its release from cactus. In a second case, dorsal, following its release from cactus in response to the ventral signal, might be phosphorylated by a kinase activity that is independent of Toll. In this latter case, the dissociation of cactus would be sufficient to stimulate phosphorylation, regardless of Toll's activation state. Stated another way, the ventral signal may regulate the phosphorylation state of dorsal by modulating the catalytic activity of a kinase or by modulating the availability of the kinase's substrate.

To examine specifically the effect of cactus dissociation on dorsal phosphorylation, we analyzed dorsal in $gd^2;cact^{A2}$ embryos. Even though signaling is blocked in these mutant embryos, we can detect a new dorsal isoform at stage 5 that comigrates with the most slowly migrating isoform in $cact^{A2}$ embryos. Thus, in these embryos dorsal is a substrate for a signal-independent kinase activity. This activity must act on free dorsal; if the signal-independent kinase could phosphorylate cactus-bound dorsal, the most slowly migrating dorsal isoform should have been present in the single $gd^2$ mutant embryos. The signal-independent kinase activity may reflect the basal activity of a Toll-activated kinase. In this case, it is only the unusual double-mutant situation (free dorsal present in the absence of signal) that allows the kinase activity to be visualized; this signal-independent kinase activity might not, therefore, be relevant in wild-type embryos. Alternatively, the signal-independent kinase activity may belong to a kinase that is wholly unregulated by Toll. Phosphorylation of dorsal by such a kinase would not convey any dorsoventral spatial information but may be required for dorsal's morphogen activity to be fully realized.

Although it is possible that all of the additional phosphorylation of dorsal in wild-type, $Tl^{10B}$, and $cact^{A2}$ embryos is catalyzed by a signal-independent kinase, the modest change in dorsal phosphorylation observed in the $gd^2;cact^{A2}$ embryos is at odds with this hypothesis. Because it is likely that a substantial fraction of dorsal is not bound to cactus in embryos carrying a $cact^{A2}$ mutation, the more slowly migrating isoforms should have dominated the $gd^2;cact^{A2}$ dorsal ensemble. Since they do not, we believe that some fraction of dorsal's signal-dependent phosphorylation is catalyzed by a Toll-dependent kinase, perhaps the product of the *pelle* gene.

The lateralized phenotype of the $gd^2;cact^{A2}$ embryos arises as a result of spatially unrestricted low-level import of dorsal into nuclei. In these embryos, a fraction of the dorsal protein may translocate into nuclei simply because it is free of cactus. Alternatively, the free dorsal may enter nuclei as a consequence of phosphorylation by the signal-independent kinase. We suggest that high-level nuclear import in wild-type embryos, however, requires additional phosphorylation by a Toll-dependent kinase. Phosphorylation of dorsal by this kinase may control the extent or steepness of the dorsal gradient.

Biochemical models describing the dissociation of the mammalian NF-κB–IκB complex have largely focused on IκB as the principal target for intracellular signals initiated by cytokines and mitogens. Such models are supported by recent experiments demonstrating the signal-dependent phosphorylation of IκB (6, 15). We have now demonstrated that the *Drosophila* counterpart of NF-κB, dorsal, also undergoes signal-dependent phosphorylation. Similar results have been reported by Whalen and Steward (55). Signal-dependent phosphorylation of dorsal is consistent with genetic data suggesting that dorsal, as well as cactus, is modified by the activation of Toll (17, 39). Taken together, the biochemical and genetic data emerging from studies of the mammalian and *Drosophila* pathways are consistent with the hypothesis that phosphorylation of both Rel-related proteins and their inhibitors is required for the proper nuclear localization and transcriptional activity of the Rel-related proteins.

## ACKNOWLEDGMENTS

We gratefully acknowledge Peter Gillespie for many critical discussions and biochemical insights during the course of this work. Angie Duke and Sherry Alexander provided excellent technical assistance. We thank Kathryn Anderson for the $tub^{R5.6}$ stock, Christiane Nüsslein-Volhard for the $Tl^{10B}$ and *dorsal* deficiency and inversion stocks, and Chris Rushlow for the pARdl expression plasmid. In addition, we thank Chris Shelton, Melanie Cobb, and Andreas Bergmann for helpful discussions and Leon Avery, Dennis McKearin, Helmut Krämer, and our colleagues in the Wasserman laboratory for comments on the manuscript.

This work was supported by grants to S.A.W. from the Robert A. Welch Foundation, the National Science Foundation, and the David and Lucile Packard Foundation. S.K.H.G. was supported by a predoctoral training grant from the National Institutes of Health.

## REFERENCES

1. **Anderson, K. V., G. Jürgens, and C. Nüsslein-Volhard.** 1985. Establishment of dorsal-ventral polarity in the *Drosophila* embryo: genetic studies on the role of the *Toll* gene product. Cell **42**:779–789.
2. **Anderson, K. V., and C. Nüsslein-Volhard.** 1984. Information for the dorsal-ventral pattern of the Drosophila embryo is stored as maternal mRNA. Nature (London) **311**:223–227.
3. **Baeuerle, P.** 1991. The inducible transcription activator NF-κB: regulation by distinct protein subunits. Biochim. Biophys. Acta **1072**:63–80.
4. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53**:211–217.
5. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB: a specific inhibitor of the NF-κB transcription factor. Science **242**:540–546.
6. **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-α: a mechanism for NF-κB activation. Mol. Cell. Biol. **13**:3301–3310.

7. **Beg, A. A., S. M. Ruben, R. I. Scheinman, S. Haskill, C. A. Rosen, and A. S. Baldwin, Jr.** 1992. IκB interacts with nuclear localization sequences of the subunits of NF-κB: a mechanism for cytoplasmic retention. Genes Dev. **6**:1899–1913.
8. **Bird, T. A., P. R. Sleath, P. C. deRoos, S. K. Dower, and G. D. Virca.** 1991. Interleukin-1 represents a new modality for the activation of extracellular signal-regulated kinases/microtubule-associated protein-2 kinases. J. Biol. Chem. **266**:22661–22670.
9. **Blank, V., P. Kourilsky, and A. Israël.** 1992. NF-κB and related proteins: rel/dorsal homologies meet ankyrin-like repeats. Trends Genet. **17**:135–140.
10. **Bomsztyk, K., J. W. Rooney, T. Iwasaki, N. A. Rachie, S. K. Dower, and C. H. Sibley.** 1991. Evidence that interleukin-1 and phorbol esters activate NF-κB by different pathways: role of protein kinase C. Cell Regul. **2**:329–335.
11. **Bomsztyk, K., B. Toivola, D. W. Emery, J. W. Rooney, S. K. Dower, N. A. Rachie, and C. H. Sibley.** 1990. Role of cAMP in interleukin-1-induced κ light chain gene expression in murine B cell line. J. Biol. Chem. **265**:9413–9417.
12. **Brautigan, D. L., and C. L. Shriner.** 1988. Methods to distinguish various types of protein phosphatase activity. Methods Enzymol. **159**:339–346.
13. **Campos-Ortega, J. A., and V. Hartenstein.** 1985. The embryonic development of *Drosophila melanogaster*. Springer-Verlag, Berlin.
14. **Chasan, R., Y. Jin, and K. V. Anderson.** 1992. Activation of the *easter* zymogen is regulated by five other genes to define dorsal-ventral polarity in the *Drosophila* embryo. Development **115**:607–616.
15. **Cordle, S., R. Donald, M. A. Read, and J. Hawiger.** 1993. Lipopolysaccharide induces phosphorylation of MAD3 and activation of c-rel and related NF-κB proteins in human monocytic THP-1 cells. J. Biol. Chem. **268**:11803–11810.
16. **Gay, N. J., and F. J. Keith.** 1991. *Drosophila Toll* and IL-1 receptor. Nature (London) **351**:355–356.
17. **Geisler, R., A. Bergmann, Y. Hiromi, and C. Nüsslein-Volhard.** 1992. *cactus*, a gene involved in dorsoventral pattern formation of *Drosophila*, is related to the IκB gene family of vertebrates. Cell **71**:613–621.
18. **Ghosh, S., and D. Baltimore.** 1990. Activation *in vitro* of NF-κB by phosphorylation of its inhibitor IκB. Nature (London) **344**:678–682.
19. **Gillespie, P. G., and A. J. Hudspeth.** 1991. High-purity isolation of bullfrog hair bundles and subcellular and topological localization of constituent proteins. J. Cell Biol. **112**:625–640.
20. **Gillespie, P. G., and A. J. Hudspeth.** 1991. Chemiluminescence detection of proteins from single cells. Proc. Natl. Acad. Sci. USA **88**:2563–2567.
21. **Gillespie, S. K. H., and S. A. Wasserman.** Unpublished data.
22. **Gordon, J. A.** 1991. Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. Methods Enzymol. **201**:477–482.
23. **Govind, S., and R. Steward.** 1991. Dorsoventral pattern formation in *Drosophila*: signal transduction and nuclear targeting. Trends Genet. **7**:119–125.
24. **Hashimoto, C., S. Gerttula, 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**:1021–1028.
25. **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.
26. **Hecht, P. M., and K. V. Anderson.** 1992. Extracellular proteases and embryonic pattern formation. Trends Cell Biol. **2**:197–202.
27. **Hecht, P. M., and K. V. Anderson.** 1993. Genetic characterization of *tube* and *pelle*, genes required for signaling between *Toll* and *dorsal* in specification of the dorsal-ventral pattern of the *Drosophila* embryo. Genetics **135**:405–417.
28. **Higgins, R. C., and M. E. Dahmus.** 1979. Rapid visualization of protein bands in preparative SDS-polyacrylamide gels. Anal. Biochem. **93**:257–260.
29. **Isoda, K., S. Roth, and C. Nüsslein-Volhard.** 1992. The functional domains of the *Drosophila* morphogen *dorsal*: evidence from the analysis of mutants. Genes Dev. **6**:619–630.
30. **Kidd, S.** 1992. Characterization of the *Drosophila cactus* locus and analysis of interactions between cactus and dorsal proteins. Cell **71**:623–635.
31. **Letsou, A., S. Alexander, K. Orth, and S. A. Wasserman.** 1991. Genetic and molecular characterization of *tube*, a *Drosophila* gene maternally required for embryonic dorsoventral polarity. Proc. Natl. Acad. Sci. USA **88**:810–814.
32. **Lindsley, D. L., and G. G. Zimm.** 1992. The genome of *Drosophila melanogaster*. Academic Press, Inc., San Diego, Calif.
33. **Lowenthal, J. W., D. W. Ballard, E. Böhnlein, and W. C. Greene.** 1989. Tumor necrosis factor α induces proteins that bind specifically to κB-like enhancer elements and regulate interleukin 2 receptor α-chain gene expression in primary human T lymphocytes. Proc. Natl. Acad. Sci. USA **86**:2331–2335.
34. **MacKintosh, C., K. A. Beattie, S. Klumpp, P. Cohen, and G. A. Codd.** 1990. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett. **264**:187–192.
35. **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 protein kinase A. Genes Dev. **6**:1654–1667.
36. **Osborn, L., S. Kunkel, and G. J. Nabel.** 1989. Tumor necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB. Proc. Natl. Acad. Sci. USA **86**:2336–2340.
37. **Pearson, R. B., and B. E. Kemp.** 1991. Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. Methods Enzymol. **200**:62–81.
38. **Rio, D. C., F. A. Laski, and G. M. Rubin.** 1986. Identification and immunochemical analysis of biologically active *Drosophila* P element transposase. Cell **44**:21–32.
39. **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.
40. **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**:1189–1202.
41. **Rushlow, C. A., 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.
42. **Rushlow, C., and R. Warrior.** 1992. The rel family of proteins. Bioessays **14**:89–95.
43. **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.
44. **Schüpbach, T., and E. Wieschaus.** 1989. Female sterile mutations on the second chromosome of *Drosophila melanogaster*. I. Maternal effect mutations. Genetics **121**:101–117.
45. **Sen, R., and D. Baltimore.** 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell **47**:921–928.
46. **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.
47. **Shirakawa, F., and S. B. Mizel.** 1989. In vitro activation and nuclear translocation of NF-κB catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. Mol. Cell. Biol. **9**:2424–2430.
48. **Shirakawa, F., U. Yamashita, M. Chedid, and S. B. Mizel.** 1988. Cyclic AMP—an intracellular second messenger for interleukin 1. Proc. Natl. Acad. Sci. USA **85**:8201–8205.
49. **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.
50. **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.

51. **Steward, R.** 1987. *Dorsal*, an embryonic polarity gene in *Drosophila*, is homologous to the vertebrate proto-oncogene, *c-rel*. Science **238:**692–694.
52. **Steward, R.** 1989. Relocalization of the *dorsal* protein from the cytoplasm to the nucleus correlates with its function. Cell **59:**1179–1188.
53. **St. Johnston, D., and C. Nüsslein-Volhard.** 1992. The origin of pattern and polarity in the *Drosophila* embryo. Cell **68:**201–219.
54. **Wasserman, S.** 1993. A conserved signal transduction pathway regulating the activity of the Rel-like proteins dorsal and NF-κB. Mol. Biol. Cell. **4:**767–771.
55. **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.
56. **Wieschaus, E., and C. Nüsslein-Volhard.** 1986. Looking at embryos, p. 199–227. *In* D. B. Roberts (ed.), *Drosophila*: a practical approach. IRL Press, Oxford.